FACTORS INFLUENCING THE EFFECTIVENESS OF RHIZOBIA IN ACID HILL SOILS

J. F. LOWE

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SUMMARY

A study has been made of some of the factors that may influence the symbiotic association of Rhizobium trifolii and Trifolium repens (white clover) in acid hill soils.

1. Significant differences between the yields of indigenous hill clover and commercial (S. 184) clover nodulated by the same strains of Rhizobium trifolii were found. Many rhizobia highly effective in nitrogen fixation on S. 184 clover were less efficient on hill ecotypes, whilst many strains comparatively ineffective on S. 184 promoted higher levels of nitrogen fixation on the indigenous material.

2. Detailed studies were undertaken of the utilization of carbohydrates by Rhizobium trifolii and of the influence of phosphate concentration, iron source and nitrogen source on the growth of the strains. Biotin and thiamin were required by all strains as growth factors.

a. Most simple sugars were rapidly metabolized and no preferential utilization of mannitol was found. Some strains did not utilize trisaccharides.

b. High phosphate concentrations were found to inhibit growth of rhizobia. No differences in requirement or of tolerance were found amongst the strains investigated.

c. As a source of iron, Fe-EDTA, which did not cause any precipitation from the medium, was found to support better growth of rhizobia than $FeCl_3$.

d. Ammonium chloride and $NaNO_3$, but not $NaNO_2$, were found to be suitable as sole sources of nitrogen.

On the basis of these investigations, a defined medium was devised. Rhizobia grown on this medium were as effective in

nitrogen fixation as controls grown on a complex medium.

3. Acid production by Rhizobium trifolii was investigated in the defined medium. The reaction of this medium frequently fell from pH 6.8 to pH 5.0 during growth. Pyruvic, acetic, propionic, butyric and iso-butyric acids were identified in the spent culture medium. The metabolic implications of this are discussed.

4. In a pot experiment, both $Ca(OH)_2$ and $Mg(OH)_2$ were used to raise the pH of a brown earth soil to 4.7 or 5.3. The addition of 0.26 M Fe-EDTA reduced clover yield at both pH levels. In the calcium-treated soil, greater yields were obtained at pH 5.3 than at pH 4.7 whilst the reverse held true in magnesium-treated soil. Rhizobia survived better in the magnesium-treated soil (pH 4.7) at the low iron treatments than in the other treatments.

5. The effects of manganese on the Rhizobium-Trifolium symbiosis were studied in greater detail.

a. Complete inhibition of growth of rhizobia was found at nutrient manganese levels greater than 900 ppm. The doubling times and rates of respiration of the strains were reduced at 200 ppm manganese. Initiation of growth of the effective strains in the presence of manganese was better than that of the ineffective strains but no differences in tolerance to the metal were observed.

b. Effective rhizobia, subcultured at fortnightly intervals for 5 months on the defined medium containing 900 ppm manganese, were shown to be less effective. They tended to regain their former efficiency of symbiosis when subcultured on the defined medium without added manganese.

c. In pot experiments it was shown that regular addition of small amounts of manganese during clover growth did not affect yields. High levels of manganese in soil for the whole period of clover growth did, however, reduce yields. The survival of all rhizobia was adversely affected by manganese at pH 5.0.

d. In agar tube and pot experiments the nodulation rate of clover by rhizobia was adversely affected by manganese, except in the case of one strain. Clover yields were also reduced.

The agronomic implications of these findings are discussed.

INTRODUCTION

In Britain, considerable attention is being given to developing methods of improving the plant productivity of hill areas, many of which comprise soils of low pH (see e.g. Muir, 1956; Simpson, 1967). In most hill areas, the application of lime and of nitrogenous and phosphate fertilizers will, in spite of the shorter growing season, greatly improve the feeding value of the herbage since more nutritious species of plants tend to replace the coarse grasses indigenous to the acid soils (Russell, 1961). However, as large areas of hill land are involved, such fertilizer treatments prove to be uneconomic. It has therefore become clear that the establishment of white clover in order to improve the nitrogen status of the soil is the most appropriate method of improving the productivity of hill pastures.

In general terms, the most important factors affecting the nitrogen-fixing capacity and, hence, the establishment and growth of white clover introduced into hill soils may be summarized under the headings: a) behaviour of rhizobia in soil; b) inherited characteristics of clover; c) soil factors; and d) climate.

a) Rhizobium as a soil saprophyte. Information concerning the genus Rhizobium has been reviewed by Allen & Allen (1950, 1958) and, most recently, by Masterson & Sherwood (1970).

The members of the genus are Gram-negative rods, aerobic and usually motile. There are currently six species within the genus although, on the basis of numerical taxonomy and DNA homology studies, a number of workers considers that fewer species are warranted

(see Dixon, 1969). Though Bisset (1952) has reported endospore formation and Gillberg (1968, 1969) heat resistant structures within the Rhizobium cell, most authorities consider that rhizobia do not produce a resting stage which enables them to survive as soil saprophytes indefinitely. Nutman (1963) reported that, in soil, the numbers of rhizobia tend to decrease with time and Jones (1966) has shown that rhizobia indigenous to acid hill soils are present in very small numbers.

The inability to survive in soil is possibly due to the nutrient requirements of these organisms. Many rhizobia have a vitamin requirement, biotin being the growth factor most commonly needed (Fahraeus & Ljunggren, 1967). However, competition with other micro-organisms for all nutrients may be a more important factor. Abdel-Ghaffar & Allen (1950) showed, in vitro, that two Streptomyces cultures were able to inhibit the growth of Rhizobium lupini and R. japonicum whilst a spore-forming bacterium was effective against many slow-growing rhizobia. Thornton, de Alencar & Smith (1949) reported S. albus and two Penicillium spp. to produce substances toxic to R. meliloti.

More recently, attention has been paid to the effects of micro-organisms on rhizobia in vivo. Holland (1966) found differences between the fungal populations of soil newly cleared of native vegetation and those of soil where Trifolium subterraneum was established. Holland & Parker (1966) reported that many of the fungi, particularly those of the genus Penicillium, produced in newly cleared soil substances both phytotoxic and inhibitory to nodulation. They suggested that, as soil ages, the fungal population changes and

becomes mainly compatible with the rhizobia and their host plant. On the other hand, Hattingh & Louw (1969) have shown that many of the bacteria found in the clover rhizoplane are antagonistic to Rhizobium trifolii, which suggests that the establishment of clover encourages the development of a microbial population antagonistic to the root nodule bacteria.

Data are also available which show that both fungi and bacteria are capable of stimulating the growth of rhizobia in pure culture and in soil (Hattingh & Louw, 1966; Sethi & Subba-Roa, 1968). The importance of this phenomenon on the survival of rhizobia in vivo cannot yet be assessed. The available evidence on these aspects of rhizobia as soil saprophytes is apparently contradictory.

Of the small population of rhizobia present in acid hill soils, a large proportion consists of strains ineffective in nitrogen fixation on commercial clovers (Thornton, 1946, 1954; MacConnell & Bond, 1957; Masterson, 1961). Holding & King (1963) suggested that the increase in the availability of metal ions, particularly those of manganese and aluminium, associated with the decrease in soil reaction, may be a factor in the development of ineffective populations. Though it has been shown in pure culture that Rhizobium effectiveness can be altered by a number of factors, including calcium deficiency (Reid, 1940; Badawy & Allen, 1963) and amino acids (Holding, Tilo & Allen, 1960; Hamdi, 1969), no data are available from soil studies to confirm these results.

b) Clover factors. The importance of the legume in the Rhizobium-legume symbiosis has been reviewed by Nutman (1956, 1969). It is

known that legumes carry various genes controlling the nodulation process. Nutman (1949) found in red clover a recessive gene which, in conjunction with a cytoplasmically transmitted factor, inhibited nodulation. A single recessive factor controlling nodulation has been reported in Glycine max (Williams & Lynch, 1954) but no control of susceptibility to infection is apparent in Medicago sativa or M. falcata (Aughtry - cited by Nutman, 1969). Nutman (1954) also reported a major gene for ineffectiveness in red clover. In all, at least four genes affecting ineffectiveness have been identified (Nutman, 1969). Bergersen & Nutman (1957) found that plants doubly recessive with one of these genes had a higher nodule count than normal plants but none of the nodules contained leghaemoglobin. Dilworth (1969) has shown, from work with yellow lupin, serradella and two strains of rhizobia, that the type of haemoglobin produced within the nodule is determined solely by the plant and not by the rhizobium.

Factors controlling early and late nodule formation and nodule number have also been reported in a number of legumes (Nutman, 1969). Jones & Burrows (1968) were able to select S. 100 white clover with a higher total nodule mass per plant. This was correlated with a higher number of nodules per plant but, as nodule size increased, efficiency of fixation, calculated on a unit-nodule basis, decreased.

Differences between clover types are marked. King (1963) has reported that white clover, indigenous to the Southern Uplands of Scotland, differs from the commercial S. 184 variety in being earlier flowering, smaller leaved and more prostrate.

Differences in the ability of clover types to tolerate differing soil conditions are also recognized. It has, for example, been shown that the response of Trifolium repens to soil calcium and manganese varies markedly from strain to strain (Vose & Jones, 1963).

c) Soil factors. The adverse effects of acid soils on legume establishment cannot be attributed solely to soil reaction but rather to nutrient deficiencies or toxicities caused by acidity (see Andrew, 1962), though Munns (1968a) has shown some stages in the nodulation of Medicago sativa to be acid-sensitive. On the other hand Small (1968) reported good nodulation of two strains of Trifolium africanum at pH 4.0. It is generally considered, however, that Rhizobium trifolii is more sensitive to pH than its host plant (Fred, Baldwin & McCoy, 1932; van Schreven, 1958).

The availability of all metals needed by legumes is affected by changes in soil reaction. Three metals in particular, iron, cobalt and molybdenum, are known to be important in the fixation process. Iron has been shown to be a constituent of the two protein fractions of nitrogenase in a variety of micro-organisms; molybdenum is associated with iron in one of these fractions (Burris, 1969). Iron is also an important constituent of the electron carrier ferredoxin, with which cobalt is also apparently associated, and of haemoglobin.

Whilst the structure of the nitrogenase system is not known, cobalt-nitrogen complexes have been chemically synthesized. It is possible that these complexes are analogous to the nitrogenase system but attempts to reduce their nitrogen to free ammonia have led

to equilibria of form:

$$CoHN_2(PPh_3)_3 + H_2 \rightleftharpoons CoH_3(PPh_3)_3 + N_2,$$

where Ph represents the phenyl group (Chatt, 1969).

Molybdenum excess is unlikely in acid soils as molybdenum is one of the few metals the availability of which increases with pH (Lucas & Davis, 1961). Manganese interferes with molybdenum uptake and this renders molybdenum deficiency more likely (Hewitt, 1958).

Cobalt has been shown to improve the growth of clover growing both on fixed and elemental nitrogen (Wilson & Hallsworth, 1965). On the other hand, toxic levels of cobalt rapidly produce iron deficiency symptoms (Hewitt, 1948).

Other metals in soil may be of indirect importance in the establishment of legumes. Calcium nutrition is complex and it is difficult to separate the effects of calcium per se from those of its salts as neutralizing agents. Little is understood of the function of calcium in the cell; it is known to be a co-factor for some enzymes (Dixon & Webb, 1964) and, probably in this capacity, is necessary for nitrogen fixation in Azotobacter (Jakobsens, Zell & Wilson, 1962). Rogers (1965) has suggested that the primary function of calcium is the maintenance of the structural integrity of cell components.

Calcium deficiency is recognized in clovers by the sudden collapse of pedicles and petioles though in lucerne and other legumes, terminal collapse occurs under such conditions. Loneragan & Dowling (1958) found hydrogen ions to depress calcium uptake by Trifolium subterraneum. Calcium and hydrogen ions were found to

affect nodulation independently above the critical values of 0.01 mM calcium and pH 4.0. The metal requirement for nodulation was higher than for growth of host plant and of Rhizobium trifolii alone (see also Hallsworth, 1958).

Norris (1958) reported calcium unnecessary for the growth of rhizobia; his data support the contention that magnesium is the essential metal micronutrient. Reid (1940), however, had previously found that rhizobia grown on calcium-free media possessed a reduced efficiency of nitrogen fixation as shown by reductions in dry weight yields and in nitrogen contents of the plants. Bergersen (1961) found evidence of a calcium requirement by rhizobia, which was confirmed by Vincent (1962). It seems likely that calcium is essential for a rigid cell wall structure in rhizobia (Humphrey & Vincent, 1962; Vincent & Humphrey, 1963) as calcium deficiency results in extreme pleomorphism (Vincent & Colburn, 1961). Doubt must therefore be expressed about Norris' (1958) findings. Magnesium is, nevertheless, important to the cell since it has been shown that low levels of the metal predispose micro-organisms to the toxic effects of other divalent cations (Abelson & Aldous, 1950). Vincent & Colburn (1961) reported that magnesium deficiency is associated with cytological abnormalities in rhizobia.

Manganese and aluminium excesses to plants are usually associated with acid soils. Both have been shown to inhibit calcium uptake in lucerne (Schmehl, Peech & Bradfield, 1952); manganese toxicity can be alleviated by additional calcium, but the effects of aluminium appear irreversible (Rorison, 1958). Little, if any, information is available concerning the effects of manganese and

aluminium on Rhizobium.

Phosphate limitation has also been incriminated as a cause of the poor establishment of legumes. Trumble & Shapter (cited by Bryan, 1962) have shown that intense competition exists between legumes and grasses for available phosphorus, which plays an important role in root and shoot development. It is also considered that nodule number and size are affected by the availability of this element (Andrew, 1962).

Munns (1965a, b & c) has shown an interaction between lime and phosphorus in cases of aluminium toxicity of Medicago sativa and Trifolium subterraneum. At levels of phosphorus adequate in the presence of lime, plants in the absence of lime grew poorly and were usually phosphate-deficient. The symptoms were more marked in the lucerne. The evidence indicates that aluminium inhibits not only calcium uptake (Schmehl et al., 1952) but also phosphate utilization.

As mentioned earlier, liming and other fertilizer applications are uneconomic in hill areas. Nevertheless, in the establishment of white clover attempts to improve the soil environment have been made. Jones & Thomas (1966) and Jones, Druce & Williams (1967) have shown that the sowing of clover seed pelleted with an effective strain of Rhizobium trifolii and with lime or with lime and superphosphate produced a significantly better sward than the use of untreated seed. Though the percentage of clover in the pellet-derived sward was lower than that in the high lime plots, it was considered that this system will prove economically justifiable since the initial costs of pelleting are much lower than those of liming whilst the maintenance requirements of both methods of reclamation are similar.

d) Climate. The effects of temperature on the Rhizobium-legume association have been recently summarized by Stewart (1966). Differences have been found in the effects of temperature on clover growing on combined and elemental nitrogen. Whilst Trifolium plants grow well on fixed nitrogen at root temperatures of 20° and 30°, inhibition of fixation occurs in nodulated plants as the temperature rises between these limits.

The data that are available suggest that low temperatures have differing effects on growth and nitrogen fixation. Russell (1961) has suggested that low temperatures in long winters may cause the shedding of many nodules in perennials such as clover. Gibson (1967), working with Trifolium subterraneum, found that the lowest temperature at which nodules would form was 7° but nodulation was retarded at all temperatures below 22°. He had previously shown (Gibson, 1963) that nitrogen fixation occurred within subterranean nodules at temperatures as low as 5°, though translocation of nitrogen to the shoots is inhibited at this temperature (Gibson, 1966).

Mes (1959) and Pate (1961) have shown that in some other legumes growth processes other than nitrogen fixation are retarded in conditions of lower temperatures whilst in other species fixation is inhibited. Such data are not available for Trifolium repens though Mence (1964) reported that a temperature of 10° is necessary for the satisfactory establishment of this species.

Altitude affects soil temperature markedly. Green (1964) suggests an average temperature lapse-rate of 1° per 150 m. of altitude. While it is common to refer to the "shorter growing season on the hill", it is probably more accurate to consider the accumulated

temperature (expressed in day-degrees) above 6^{o}, the threshold of vegetative growth for most plants. Since altitude has a large effect on temperature, it can easily be seen how the accumulated temperature above 6^{o} drops rapidly, thus resulting in a large decrease in plant productivity at high altitudes as compared with sea-level. Further, it can be seen that temperature may be indirectly reducing the nitrogen status of the acid hill soils due to the higher threshold of white clover vegetative growth reported by Mence (1964).

Day length (Masefield, 1958; Gibson, 1967) and light intensity (van Schreven, 1958) have been shown to be important in the nodulation of legumes. Day length has also been investigated for its effects on yields of legumes growing in the presence of toxic levels of manganese and aluminium (Rorison, Sutton & Hallsworth, 1958). These authors consider that at low aluminium concentrations, climate is important in influencing the effects of the metal; conversely, with manganese the quicker the growth, the quicker the toxicity symptoms appear.

Little attention has been focussed on the quality of light. Lie (1969) has shown that red light slightly increases the number of nodulated plants but decreases the numbers of nodules per plant; this effect is counteracted by far-red light. Though the proportion of red in sunlight is higher in spring than later in the year, it is moot whether this effect significantly assists the nodulation of clover and other legumes.

Moisture regime has also been found to affect the legume symbiosis. Singer (1964) and Masterson (1968) found significant differences between efficiencies of rhizobia isolated from wet and

dry sites; this difference was still apparent after lime and fertilizer treatments, though the mean levels of effectiveness had risen (Masterson, 1968).

Waterlogging of soils results in several chemical changes. Anaerobiosis, which results from waterlogging, causes an increase in the availability of a number of metals but decreases the amounts of available phosphorus and nitrate (McLaren & Skujins, 1967). Patrick & Turner (1968) have reported that increase in manganese availability is one of the first measurable effects of the reducing conditions caused by waterlogging. Ng & Bloomfield (1962) found that manganese mobilised by flooding remained equally available after aeration of the flooded soil. Cobalt and zinc behaved similarly but in general the reaction was reversible.

The study described here was designed to investigate some of the characteristics of rhizobia indigenous to acid hill soils and that might explain the predominance of Rhizobium strains ineffective in nitrogen fixation.

The work is described in five parts:-

1) As little information is available about the relationship of indigenous white clover to commercial varieties, a comparison of the effectiveness of hill rhizobia on the two clover types was made;

2) A defined medium for the growth of Rhizobium trifolii was developed so that studies could be undertaken of the effects of metals on the growth of R. trifolii;

3) Growth of R. trifolii in the defined medium

was accompanied by a decrease in pH. Identification of the acids produced was therefore undertaken;

4) The data available in this laboratory suggested that iron-chelate treatment of soil planted with white clover might assist in the establishment of the legume in more acid conditions. The effect of this treatment on the growth of S. 184 clover and on the survival of the root nodule bacteria was therefore studied;

5) After screening a number of metals for their effects on the growth of R. trifolii in pure culture, a study was made of the effects of one of these, manganese, on the Rhizobium-Trifolium symbiosis.

No investigations have been undertaken of the effects of climate, nor of other clover and soil factors. Nevertheless it is appreciated that the results of the work described herein cannot be divorced from these other factors if the problems associated with hill pasture clover establishment are to be fully understood.

MATERIALS AND METHODS

Source of rhizobia.

The 20 strains used throughout this work were isolated from Trifolium repens growing in soils as detailed in Table 1.

Two substrains, P3R and P3S, derivatives of strain P3, were also used. P3R, the rough derivative, tended to revert to smoothness.

Stock cultures were regularly subcultured on a mannitol yeast extract salts agar (medium 79 - Fred & Waksman, 1928), incubated for 4 days at 27° and stored in the refrigerator at 4°. Three-day-old cultures, grown at 27° on the same medium, were used for experimental purposes.

Source of clover.

British certified Aberystwyth S. 184 Wild White Clover was normally used. In one experiment, seed obtained from Dr Alan Smith of the Scottish Plant Breeding Station was also used. This material was prepared by growing indigenous hill white clover ecotypes in insect-proofed glasshouses free from commercial pollens. Pollination was by segregated bees.

Media used.

a) Routine bacteriological media. For the routine maintenance and the preparation of cultures, a modification of Fred & Waksman's (1928) medium 79 was used. This modification had the following composition: mannitol, 10.0 g.; yeast autolysate, 25.0 ml.; $CaCO_3$, 3.0 g.;

TABLE 1.

Origin of rhizobia.

Culture no.	Source		pH	Isolated by
	Locality	Soil type		
1, 2, 6, 7	Sourhope, Roxburghshire	Brown earth	5.5	A. J. Holding
P1, P2, P3	Lewis	Improved peat	8.0	
FA6	Upper Fulford Edinburgh	Improved hill soil	6.0	
W19	Aberystwyth	Hill soil		D. G. Jones
7S	Sourhope	Brown earth	5.6	M. Singer
8			5.7	
1DL			5.6	
9CS			5.7	
6CL			6.0	
3AS			5.6	
4AL			5.7	
7AL			5.6	
8AL			5.7	
8CS			5.7	
9CL			5.7	
P3R, P3S	Substrains of P3, isolated from plate culture			

K_2HPO_4, 0.5 g.; $MgSO_4.7H_2O$, 0.2 g.; NaCl, 0.1 g.; Fe-EDTA ("Sequestrene Iron Complex CP2" - Johnson's of Hendon Ltd, London, N.W.4), 0.2 g.; "Oxoid" No. 3 agar, 12.0 g.; deionized water to 1 l.; pH 6.8. The yeast autolysate was prepared by the method of Gibson, Stirling, Keddie & Rosenberger (1958).

A completely synthetic medium (medium FB), based on that of Bergersen (1961), was used in some experimental work. This had the following composition: glucose, 5.0 g.; NH_4Cl, 1.0 g.; Na_2HPO_4, 0.1 g.; $CaCl_2$, 0.04 g.; $MgSO_4.7H_2O$, 0.1 g.; Fe-EDTA, 0.2 g.; biotin, 250 µg.; thiamin, 100 µg.; deionized water to 1 l.; pH 6.6. "Oxoid" No. 3 agar (12.0 g.) was added when a solid medium was required. Filter sterilized glucose and vitamin solutions and autoclaved phosphate solution were added aseptically after the rest of the medium had been sterilized.

b) Routine plant growth medium. Plants were grown on Bond's modified Crone's agar of the following composition (Allen, 1951): KCl, 317 mg.; $Ca_3(PO_4)_2$, 180 mg.; $CaSO_4.2H_2O$, 137 mg.; $MgSO_4.7H_2O$, 55 mg.; $Fe_2(SO_4)_3.9H_2O$, 27 mg.; $CuSO_4.5H_2O$, 5 mg.; $MnSO_4.4H_2O$, 12 mg.; H_3BO_3, 5 mg.; K_2HPO_4, 268 mg.; "Oxoid" No. 3 agar, 12.0 g.; deionized water to 1 l.; pH 6.5.

Following the recommendations of Anderson (1956), Ahmed & Evans (1960) and Hewitt (1966), molybdenum (0.02 m.e./l. as $Na_2MoO_4.2H_2O$) and cobalt (0.0002 m.e./l. as $CoSO_4.7H_2O$) were added to the medium.

Twenty-five ml. of the medium were dispensed into 200 x 25 mm. "Pyrex" tubes and uniformly sloped before the agar solidified.

All media and separate constituents, except where noted, were autoclaved either momentarily at 128° or for 15 min. at 121°.

Estimation of effectiveness of Rhizobium strains.

Seeds of Trifolium repens were surface sterilized by immersion in 95 p.c. alcohol and 0.1 p.c. (w/v) $HgCl_2$, both for 3 min. (Allen, 1951). The seeds were thoroughly washed in sterile water and dried overnight before being spread on agar plates of medium 79 to confirm microbiological sterility. The seed was germinated at 27° in the dark for 4 days.

Three germinated, uncontaminated seedlings were then transferred to each slope of the plant growth agar. The tubes were placed in wooden racks that shaded the seedlings' roots and then put in the greenhouse for 1 week to enable the plants to be checked for sterility and health, both of which factors seemed to vary with seed batch.

One ml. of a fairly turbid suspension of the test strain of Rhizobium trifolii in sterile deionized water was pipetted over the root hairs of the 3 seedlings.

After a 10 week growth period, the clover was harvested. Wet weights (shoot plus root), which Singer (1964) found to be closely correlated with dry weights, were recorded in all experiments.

Acid hill soil.

For pot experiments, soil was obtained from the Sourhope area of the Cheviot Hills. It was an oligotrophic brown earth derived from glacial drift, in turn derived from Andesitic larva. It is characterized by the dominance of Festuca ovina, Deschampsia flexuosa and Nardus stricta (King, 1962). It has the

following mineral status:

0.5 N acetic acid extractable (ppm)	Co : 0.031	
	Ni : 0.140	
	Pb : 0.976	
	Zn : 11.0	
Total (ppm)	Cu : 24.0	
	Mg : 0.74	
mg. exchangeable/ 100 g. air dry soil	K : 16.1	
	Ca : 11.0	
	Mg : 7.1	
	Mn : 5.7	pH : 4.2

These values are quite normal for this soil type with the exception of the cobalt value which is unusually low. Muir (1956) reports that the percentage loss on ignition of the A horizon of the Sourhope soils is about 11 p.c. (high) whilst the soluble phosphate, obtained by acetic acid extraction, is about 4 mg./100 g. soil (low).

Soil was taken from the 0 - 300 mm. layer and sieved through a 6 mm. screen before use. Each sample was amended with 0.19 g. $Na_2HPO_4.2H_2O$/kg. oven-dried soil (equivalent to 84 kg. P/ha.) and 1.0 g. KCl/kg. oven-dried soil (equivalent to 117 kg. K/ha.). Each sample was also limed with $Ca(OH)_2$ to pH 4.5 or 5.0 as required, potted and kept for a few weeks to allow equilibration of the soil at the desired pH. During this period, the moisture regime was kept constant by daily watering. Each pot was then inoculated with an aqueous suspension of the test strain of Rhizobium trifolii to give a count of approximately 400 rhizobia/g. of soil and sown with sterile clover seedlings.

Estimation of numbers of rhizobia in soil.

The numbers of Rhizobium trifolii in a soil sample were determined by the method of Brockwell (1963), in which dilutions of soil are inoculated over the roots of sterile clover seedlings growing on nitrogen-free agar. The aggregates of soil and bacteria were disrupted by mixing 10 g. soil with 100 ml. quarter strength Bond's modified Crone's liquid medium in an "Osteriser" (John Oster Mfg Co., Milwaukee, Wisconsin, U.S.A.) for 1 min.

Growth curves of rhizobia.

Growth was followed in an "Eel" nephelometer head used in conjunction with an "Eel" Unigalvo type 20 (Evans Electroselenium Ltd, Halstead, Essex). The nephelometer readings were calibrated by obtaining viable counts (Miles & Misra, 1938) of samples of known turbidity.

The growth curves were checked against dry weight determinations of the cultures at 105^{o} to ensure that the doubling times derived from nephelometry were reliable.

Acid production by rhizobia.

Three-day-old cultures of Rhizobium trifolii were transferred to slopes of Norris' (1965) mannitol yeast extract medium. Yeast autolysate (25.0 ml.), prepared by the method of Gibson et al. (1958), was substituted for the yeast extract of the original recipe. "Oxoid" No. 3 agar (12.0 g./l.) was the solidifying agent.

Cultures were inspected at 3, 7 and 28 days for acid production. The final pH was determined by adding 10 ml. deionized

water to each culture and leaving overnight. The reaction was determined by means of a "Pye" model 79 pH meter.

Determination of organic acids.

Pyruvate was determined by the method of Friedemann & Haugen (1943) in which the acid is treated with 2, 4-dinitrophenylhydrazine and the resultant hydrazone separated by treatment with first benzene and then Na_2CO_3 solution.

Carboxylic acids were identified in acidified spent medium by the method of Rogosa & Love (1968). The determination was made in a "Pye" Series 104 gas liquid chromatograph with a type 2 flame ionization dual detector head. The 1.52 m. columns were packed with "Phasepak Q", 85 - 100 mesh, a polyaromatic resin consisting of polystyrene cross-linked with divinyl benzene. Before packing, the column material was treated with "Trimer Acids" in acetone. (Both "Phasepak Q" and "Trimer Acids" were supplied by Phase Separations Ltd, Deeside Industrial Estate, Queensferry, Flintshire.).

Oxygen-free nitrogen (British Oxygen Co.) was used as the carrier gas, flow rate 80 ml./min. The oven temperature was 146°.

Under these conditions, the retention times of the carboxylic acids, measured from the start of the injection peak, were as follows: acetic, 6 min.; propionic, 11 min.; iso-butyric, 18 min.; and butyric, 21 min.

Determination of respiration rates of rhizobia cultures.

Respiration rates were determined using the "YSI" model 153 Biological Oxygen Monitor (Shandon Scientific Co., 65 Pound

Lane, London, N.W.10). Three ml. samples of test suspensions were equilibrated in cuvettes at 27° for 3 min. before the probe was inserted; the respiration of each suspension was measured over 10 min. and the rate of respiration calculated from the slope of the trace of the multivolt recorder.

Statistics.

Throughout this report, the following convention is followed:

Not significant		NS
Significant	P = 0.05	*
Highly significant	P = 0.01	**
Very highly significant	P = 0.001	***
Standard Error		SE

EXPERIMENTAL WORK.

PART I : A COMPARISON OF THE EFFECTIVENESS OF HILL CLOVER RHIZOBIA ON COMMERCIAL AND INDIGENOUS CLOVER ECOTYPES

Many soil, climatic, plant and bacterial characteristics are known to influence the nitrogen fixation process that occurs in the Rhizobium-leguminous plant association. Relatively little attention, however, has been devoted to the growth, survival and effectiveness in nitrogen fixation of rhizobia under the ecological conditions found in nature.

It is known that the Rhizobium populations of the root nodules of hill clover ecotypes are predominantly ineffective on commercial clovers. Information is here presented to show whether these indigenous Rhizobium populations are equally ineffective on the native hill clovers.

Methods.

The responses of commercial (Aberystwyth S. 184) and hill clovers to inoculation with 20 strains of Rhizobium trifolii were compared on agar slopes (see "Materials and Methods"). Four replicates of each treatment, in a pure random design, were used.

Results and Discussion.

The results (Table 2 and Appendix 1) showed marked differences in the responses of the two seed types to the same rhizobia. In terms of total wet weight, the strains that were effective on S. 184 clover promoted less growth on the indigenous material, whilst those ineffective on commercial seed produced a

TABLE 2.

The response of S. 184 and indigenous hill ecotype clovers to inoculation with *Rhizobium trifolii*.

Plant yield (wet wt) mg./tube of 3 plants (mean of 4 replicates)

Rhizobium Strain	Clover ecotypes S. 184		Indigenous
		SE: ±35	
FA6	661		372
P3	610		405
9CS	567		462
1DL	510		526
P1	480		383
1	442		436
P2	433		364
8AL	430		429
8	427		399
9CL	304		456
7AL	287		289
6CL	260		112
3AS	253		379
7	247		341
6	218		389
8CS	212		237
4AL	200		404
7S	169		267
W19	102		100
2	79		92
Means	344	SE: ± 8	342
Uninoculated controls	80		77

better response on the wild type. The indigenous ecotypes, growing under good conditions, are much smaller than the S. 184. King (1963) reported the indigenous white clover populations to be earlier flowering, smaller leaved and more prostrate than S. 184. However, data are not available to show whether the smaller response of the indigenous material to the effective rhizobia results from nitrogen fixation limitation or from genetic control of other growth processes.

Nutman (1954) has shown that effectiveness is genetically controlled in red clover and it is not unreasonable to assume that in white clover too effectiveness is under the control of the plant genome. Strain 4AL is the most interesting strain in this respect, being highly effective on indigenous clover but relatively ineffective on S. 184. Dixon (pers. comm.) found that, whilst the early stages of infection by strain 4AL are very similar in both clovers, there is a premature breakdown of bacteroids in the nodules of S. 184 plants. Rod-shaped rhizobia, which remain in considerable numbers in the infection thread, then enter the nodule region and are to be found in large numbers throughout the full-grown nodule, apparently not contributing to nitrogen fixation.

Dixon also observed that nodules of 4AL on S. 184 plants contained little, if any, haemoglobin whilst those on indigenous clovers were rich in the protein. This suggests that, though haemoglobin production is controlled by the host plant (Dilworth, 1969), the operation of this gene is dependent on some property, as yet unknown, of the bacteroids or on another plant gene.

Table 2 shows that the difference between the means of the yields was not significant. However, it cannot be deduced

that the nitrogen-fixing ability of the Rhizobium hill population is independent of clover type as the strains used were not randomly selected and there is a higher proportion of rhizobia ineffective on S. 184 in natural populations.

In Table 3 the yields are tabulated as percentages of the yields of strain FA6. If the yields obtained represent the maximum nitrogen fixation possible by these symbioses over 10 weeks; and if one regards a yield of over 50 p.c. as an effective response, 9 of the 20 strains (45 p.c.) are effective on S. 184 clover whereas 16 (80 p.c.) are effective on the indigenous material.

Holding & King (1963), using a height grading system to estimate approximate effectiveness, considered only 27 p.c. of isolated hill rhizobia to be effective on S. 184 clover. This figure accords well with that of Thornton (1946) and other workers. These results, however, support the view of Norris (1965) that this method of assessing effectiveness may not be a fair reflection of the nitrogen-fixing ability of indigenous rhizobia in symbiosis with native clovers. These results suggest an effective hill population of at least 65 p.c. and the contribution of white clover to hill pastures must, therefore, be higher than has previously been recognized.

However, the practice of testing Rhizobium isolates on commercial seed remains important. Attempts to improve hill land are made with this seed and it is therefore necessary to know the type of response expected from the indigenous bacterial population. A very good response to the sowing of commercial seed would seem feasible if a few effective strains of rhizobia are well established.

TABLE 3.

The response of S. 184 and indigenous hill ecotype clovers to inoculation with *Rhizobium trifolii*.

P.c. yield compared with strain FA6

Rhizobium strain	Clover ecotypes	
	S. 184	Indigenous
FA6	100	100
P3	92	109
9CS	86	124
1DL	77	141
P1	73	103
1	67	117
P2	66	98
8AL	65	115
8	65	107
9CL	46	123
7AL	43	78
6CL	39	30
3AS	38	103
7	37	92
6	33	104
8CS	32	64
4AL	30	108
7S	26	79
W19	15	30
2	12	25

EXPERIMENTAL WORK.

PART II : FACTORS AFFECTING GROWTH OF RHIZOBIA IN SYNTHETIC MEDIA

For the maintenance of rhizobia, a complex medium such as mannitol yeast extract agar is ideal but where a study of the effects of individual constituents on growth is to be made, such a medium is unsatisfactory.

Attention has already been drawn (see "Introduction") to the controversy regarding the importance of calcium in the nutrition of rhizobia. Differences of opinion also exist regarding other growth characteristics. With a view to developing a suitable synthetic medium for the growth of rhizobia, a number of factors was investigated; biotin and thiamin were required by all strains.

a. Utilization of carbohydrates by Rhizobium trifolii.

In the literature, differing opinions are to be found as to whether carbohydrates are oxidized or fermented by rhizobia. The majority of workers (see Bergey, 1957) claims that monosaccharides are rapidly oxidized. Moffett & Colwell (1968), however, reported that fast-growing rhizobia metabolized glucose fermentatively. Ishizawa (cited by Graham, 1964) also reported fermentation of carbohydrates by rhizobia.

Methods.

Three-day cultures of Rhizobium trifolii (20 strains) were inoculated into Hugh & Leifson's (1953) medium with glucose or lactose as carbon source. After 4 weeks, only 4 strains, P3, W19,

8 and P2, had grown oxidatively in the glucose medium and none fermentatively. Further tests showed that the indicator, bromothymol blue, was toxic to these rhizobia at the concentration recommended by Hugh & Leifson (1953).

To avoid this and the possible masking of acids from deamination products of peptone, Clark's (1966) modification of Hugh & Leifson's medium, which had a lower organic nitrogen and indicator content, was used. This medium had the following composition: casamino acids, 1.0 g.; NaCl, 0.5 g.; K_2HPO_4, 0.5 g.; $MgSO_4.7H_2O$, 0.1 g.; bromothymol blue, 0.003 g.; "Oxoid" No. 3 agar, 1.5 g.; deionized water to 1 l.; pH, 6.8; the carbon source (5.0 g.), Fe-EDTA (0.025 g.) and $CaCl_2$ (0.04 g.) were added aseptically after sterilization. Seventeen sugars and sugar derivatives were used, as shown in Table 4.

The cultures were inspected at 3, 7, 14 and 28 days after inoculation.

Results and Discussion.

The results (Table 4) provide no evidence to support the results of Moffett & Colwell (1968), which show that fast-growing rhizobia are able to ferment glucose with the production of acids. In many tubes, acid, as recognized by indicator change, was observed throughout the medium but this had diffused from the surfaces of cultures, to which growth of rhizobia was confined. With the trisaccharides, no growth occurred at the surfaces of the cultures but bands of growth developed at 2 mm. to 5 mm. below the surfaces, suggesting that rhizobia require a reduced oxygen tension when utilising complex carbohydrates. No strains were consistently

TABLE 4.

Utilization of carbohydrates by Rhizobium trifolii.

Carbohydrates	Time (days) 3	7	14	28
Glucose	20‡			
Galactose	20			
Arabinose	20			
Mannose	20			
Mannitol	20			
Rhamnose	19	19	19	19
Xylose	20			
Sorbose	19	19	19	19
Fructose	15	15	15	15
Sucrose	16	16	16	17
Lactose	8	8	9	9
Maltose	12	15	17	20
Cellobiose	6	11	12	12
Raffinose	14	17	17	17
Melezitose	5	5	6	6
Melibiose	13	16	16	16
Salicin	18	19	19	19
No carbohydrate	1	1	2	2

‡ Number of strains/20 oxidising carbohydrates.

negative in their ability to metabolize carbohydrates.

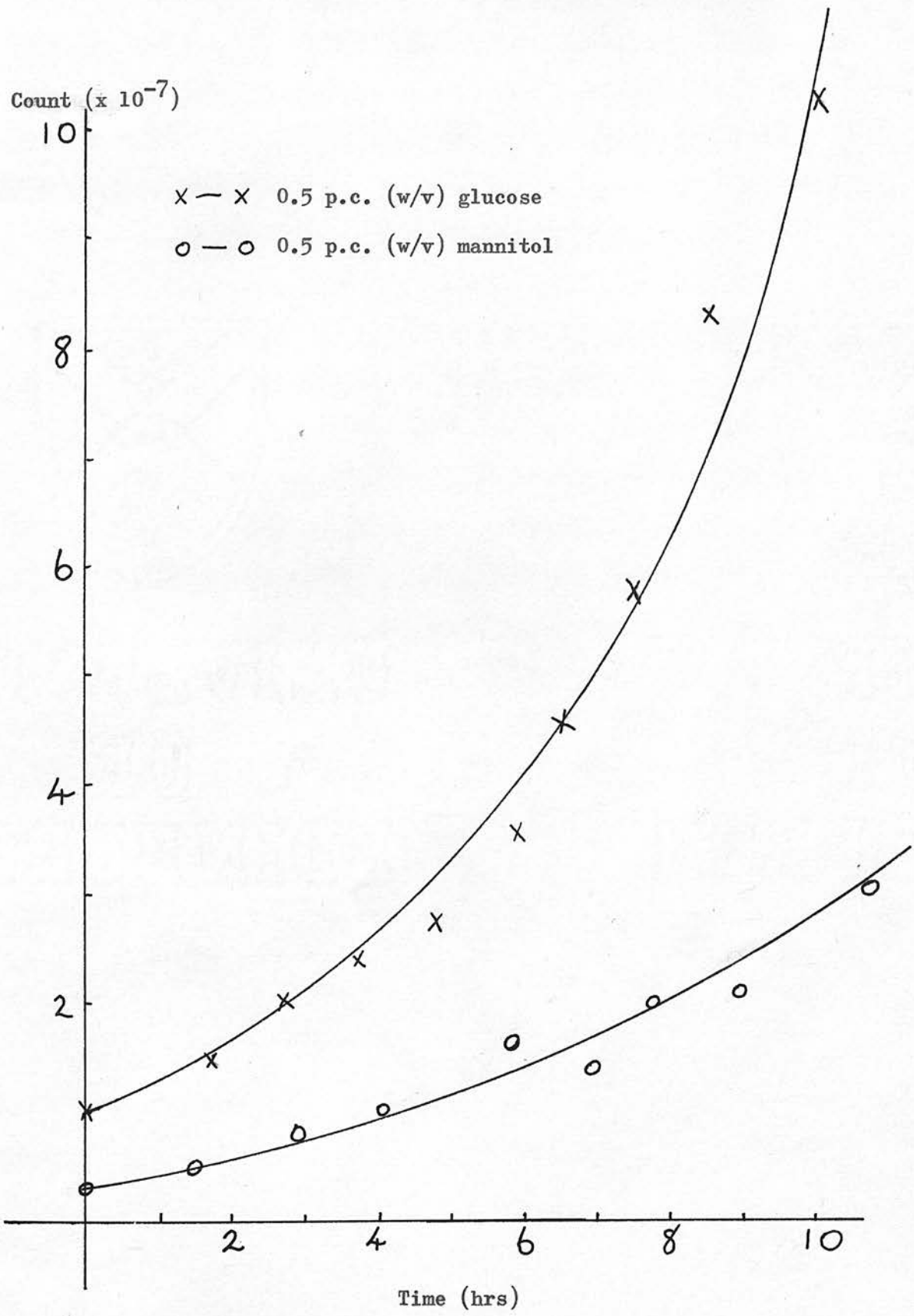
These results are mainly in accord with those of Graham (1964) who, using an agar plate technique to test for carbohydrate utilization, showed Rhizobium trifolii to metabolize many sugars. They differ, however, in that Graham (1964) reported all his strains of R. trifolii to utilize lactose whereas less than half the strains investigated in this study could grow on the substrate (Table 4).

These findings also differ from those of Moffett & Colwell (1968) in the inability of these strains to grow in Hugh & Leifson's (1953) medium. It could well be that there is wide variation in the sensitivity of rhizobia to bromothymol blue and other indicators.

No evidence was obtained to suggest preferential utilization of any monosaccharide by these organisms. All hexoses were rapidly utilized though rhamnose was not metabolized by strain 1DL. Growth experiments (see "Materials and Methods") with glucose and mannitol as sole sources of carbon and energy in Bergersen's (1961) medium showed that growth on 0.5 p.c. (w/v) glucose was as rapid as that on 0.5 p.c. (w/v) mannitol (Figure 1).

FIGURE 1.

The effect of carbohydrate source on the growth of Rhizobium trifolii.



b. The effects of phosphate concentration and iron source on the growth of Rhizobium trifolii.

For the purpose of studying, in vitro, the effects of some soil factors on the growth of Rhizobium trifolii, it was at first considered that Bergersen's (1961) medium would be suitable. However, in the preparation of this medium, precipitation occurred when the sterile constituents were mixed together. The precipitate was thought to be ferric orthophosphate, which is only slightly soluble in water. This rendered the medium unsuitable for use because of the possibility that essential micronutrients might co-precipitate with the phosphate (Reischer, 1951).

Chelates, which form stable water-soluble complexes, can be used to control the amounts of a metal in solution (Martell, 1957). They act as "metal buffers", so that only a small proportion of the total metal content is free in solution at any one time.

Chelating agents may either enhance or inhibit the growth of micro-organisms. Some chelating agents are normally toxic to bacteria. 8-Hydroxyquinoline, for example, forms essentially irreversible complexes with metals (Albert, Rubbo, Goldacre & Balfour, 1947). On the other hand, Lankford, Kustoff & Sergeant (1957) showed that metals essential for growth are more available after chelation by assimilable compounds. In particular, EDTA has been shown to be satisfactory for microbial culture (Hutner, Provasoli, Schatz & Haskins, 1950; MarciasR & Eppley, 1963).

Attempts were therefore made to modify Bergersen's (1961) medium by selecting a source of iron which did not precipitate with phosphate and by varying the concentration of phosphate used.

Methods.

Bergersen's (1961) medium, with the substitution of 0.5 p.c. (w/v) glucose for 1.0 p.c. (w/v) mannitol, was used in this investigation.

To study the effect of phosphate on growth, 6 concentrations were used viz. 0.1 M, 0.05 M, 0.01 M, 0.005 M, 0.001 M and 0.0005 M. A phosphate-free control was also incubated.

To study the effect of iron source on growth, the concentration of $FeCl_3$ in the medium was varied in the presence of different concentrations of Na_2EDTA. Fe-EDTA was also tested as a source of iron. The concentrations of the compounds are detailed in Table 5.

TABLE 5.

Concentrations (mM) of iron used in iron source experiments.

Iron source	Medium A	B	C	D	E	F	G	H
$FeCl_3$	0.2	0.1	0.2	0.1	-	-	-	-
Na_2EDTA	0.2	0.5	0.5	1.0	-	-	-	-
Fe-EDTA	-	-	-	-	1.0	0.5	0.1	-

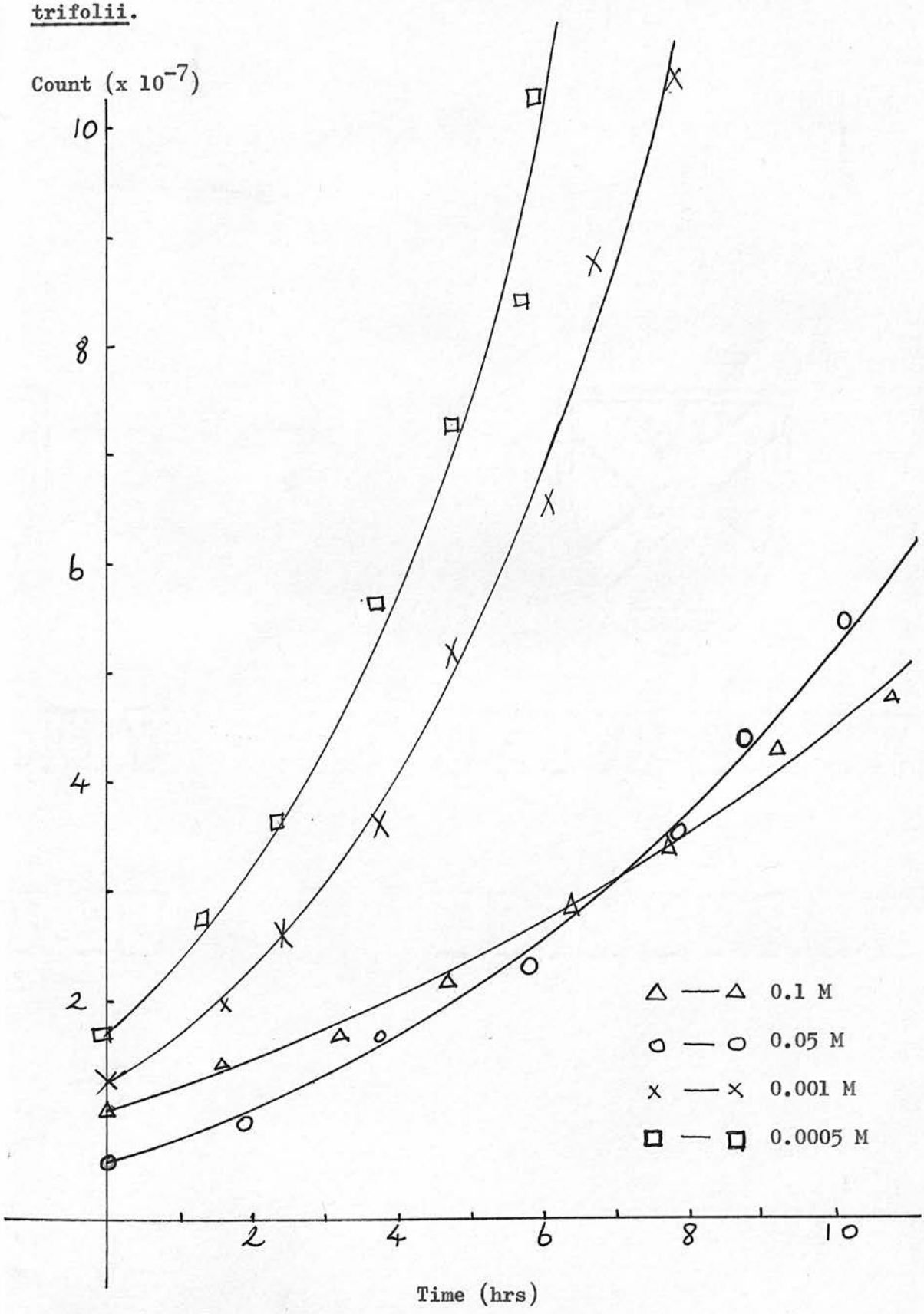
Growth curves were obtained as described in "Materials and Methods".

Results and Discussion.

Growth of Rhizobium trifolii was inhibited at high phosphate concentrations (Figure 2). The slowest doubling time was found with

FIGURE 2.

The effect of phosphate concentration on the growth of Rhizobium trifolii.



0.1 M phosphate; doubling time was also reduced with 0.05 M phosphate. No growth was observed in the absence of phosphate.

No information concerning the phenomenon of phosphate inhibition of growth is readily available. Whittenbury (pers. comm.), however, has observed that the growth of methane-utilizing bacteria is inhibited by high concentrations of nutrient phosphate.

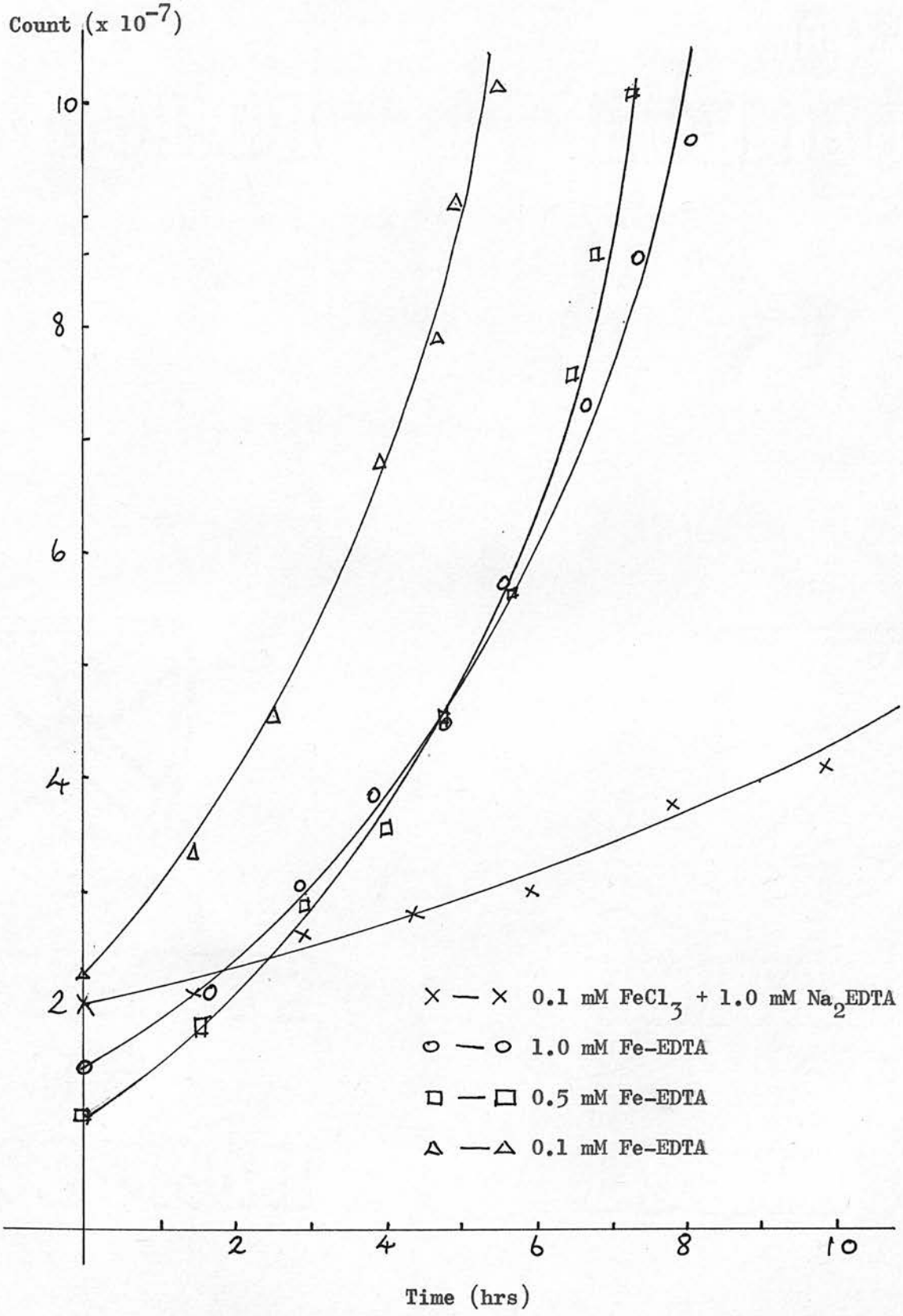
These results support the choice of 0.001 M phosphate by Bergersen as the most suitable concentration for the growth of rhizobia. However, the medium still tended to precipitate, particularly after a few days' incubation.

In the iron source experiments, precipitation of constituents occurred in media A, B, C and, occasionally, D, where the EDTA content was much greater than the iron content of the medium. No precipitates were observed in media E, F and G, those with Fe-EDTA as the iron source.

Typical growth curves of Rhizobium trifolii growing in these media are shown in Figure 3; no growth was observed in the iron-free control (medium H).

FIGURE 3.

The effect of iron source on the growth of Rhizobium trifolii.



c. Growth of Rhizobium trifolii on inorganic nitrogen compounds.

Bergersen (1961) reported that the use of sodium glutamate as a nitrogen source gave higher growth yields of rhizobia, faster growth rates and shorter lag phases in comparison with inorganic nitrogen sources. However, glutamate is, in common with other amino-acids, a chelating agent (Albert, 1950; Harris & Livingstone, 1964) and it was considered that this might mask metal effects on rhizobia. On this account, an inorganic source of nitrogen seemed more suitable.

Methods.

Three sources of inorganic nitrogen were investigated, NH_4Cl, $NaNO_3$ and $NaNO_2$ (concentration 0.02 M).

Growth curves were obtained as described in "Materials and Methods".

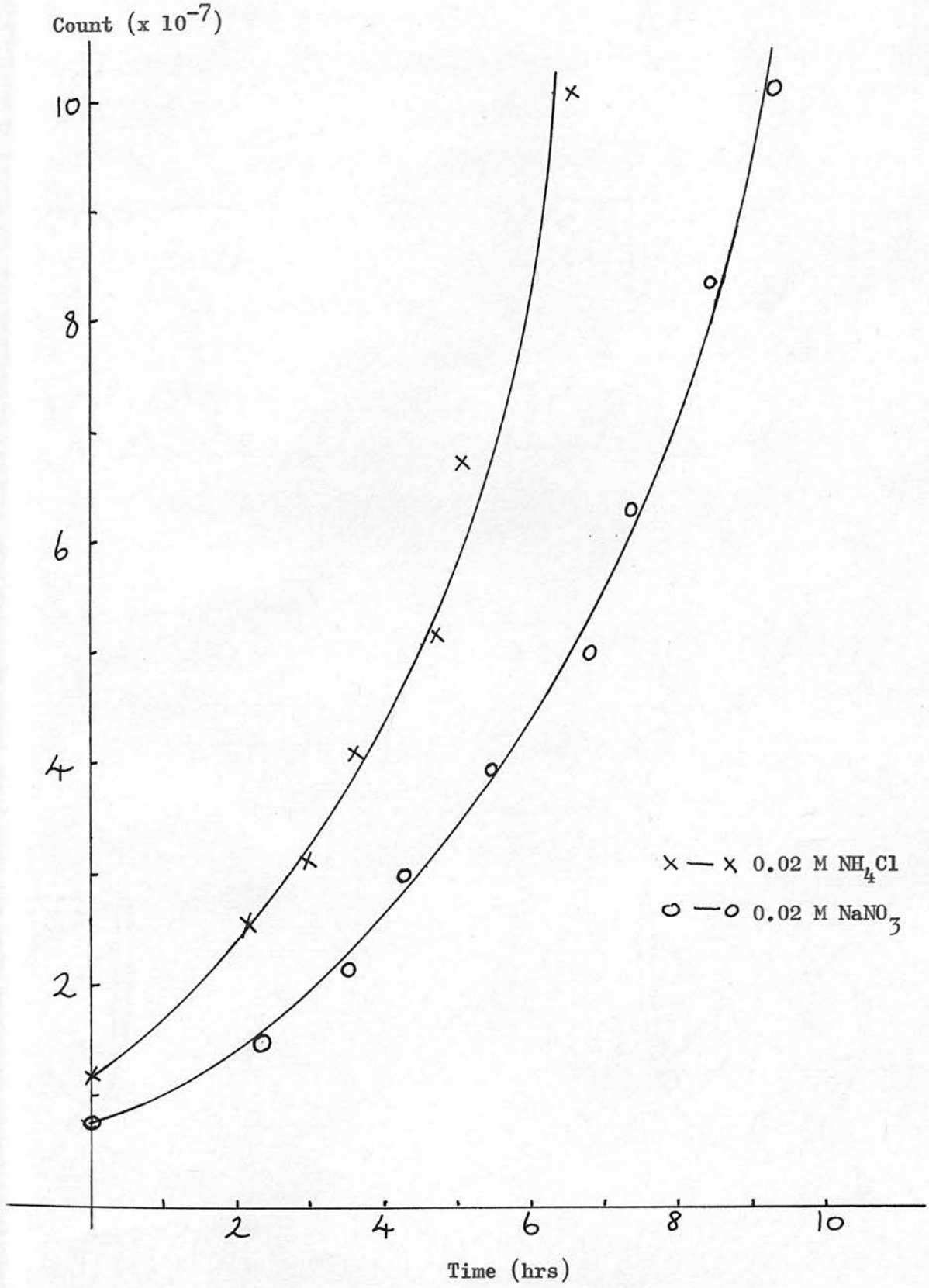
Results and Discussion.

No growth was obtained with nitrite as a substrate. With both NH_4Cl and $NaNO_3$ satisfactory growth was obtained (Figure 4). Comparable growth rates were obtained on the two nitrogen sources but, contrary to the finding of Bergersen (1961), no large differences in the lag phases were observed with rhizobia utilizing the two substrates.

It was decided to use NH_4Cl as sole nitrogen source in the defined medium.

FIGURE 4.

The effect of nitrogen source on the growth of Rhizobium trifolii.



d. A comparison of the effectiveness on commercial clover of *Rhizobium trifolii* after growth on complex and defined media.

Media for rhizobia must support good growth and not cause attenuation of the organism.

It has been shown by a number of workers that the growth medium for *Rhizobium* spp. can markedly reduce effectiveness. Holding, Tilo & Allen (1960) found a reduction in effectiveness when *R. meliloti*, *R. trifolii* and *R. leguminosarum* were cultured on amino-acid-rich media; Hamdi (1968, 1969), working with *R. meliloti*, has confirmed the work. It had previously been shown that prolonged cultivation of rhizobia on glycine media reduced first effectiveness and then infectiveness (Longley, Berge, van Lanen & Baldwin, 1937; Wolf & Baldwin, 1940).

On the other hand, calcium-deficiency has been shown to reduce effectiveness (Reid, 1940) and it is of interest that Bergersen (1961) records, in a footnote, that the *Rhizobium* strains used by Norris (1959) to demonstrate a magnesium-requirement were found to be of changed symbiotic character after maintenance on synthetic media.

It was therefore considered that the modified medium (medium FB, see "Materials and Methods") should be tested for any effect it might have on the *Rhizobium-Trifolium* symbiosis.

Methods.

Nine strains of *Rhizobium trifolii* were subcultured on medium FB at 2-weekly intervals for 5 months. Concurrently, they were similarly subcultured on medium 79.

All cultures were then inoculated over the roots of

S. 184 seedlings on agar slopes, arranged in a pure random design (6 replicates).

Wet weights were recorded after 10 weeks' growth.

Results and Discussion.

The results (Table 6 and Appendix 2) show an insignificant medium-strain interaction. It was therefore considered that medium FB was satisfactory for the culture of Rhizobium trifolii.

Medium FB was subsequently used in the work reported here. No problems were found in its preparation.

TABLE 6.

The response of S. 184 white clover to inoculation with Rhizobium trifolii after growth on media FB and 79.

Plant yield (wet wt) mg./tube of 3 plants (mean of 6 replicates)

Rhizobium strain	Media FB		Media 79
		SE: ±44	
P3	591		476
P3S	534		531
P3R	600		544
6CL	523		457
8	259		112
8AL	132		136
7S	112		75
3AS	84		108
2	58		68
Mean	321	SE: ±15	279

EXPERIMENTAL WORK.

PART III : ACID PRODUCTION BY RHIZOBIUM TRIFOLII

Holding & King (1963) suggested that the increased availability of metal ions at acid pH's may be an important factor in the development of ineffective hill populations of Rhizobium trifolii. Norris (1965) found that Rhizobium isolates from non-acid soils excreted acidic materials into their growth medium. He considered this a defensive mechanism, designed to keep the environmental reaction approximately neutral, and deduced that rhizobia from acid environments should excrete alkaline materials to the same end.

An investigation of this hypothesis was undertaken.

Methods.

Acid production by rhizobia was assessed by the method of Norris (1965), briefly described in "Materials and Methods".

Acid production by rhizobia growing in medium FB was also investigated. Ammonium chloride and $NaNO_3$ were both used as nitrogen sources. Five ml. samples were aseptically withdrawn from 250 ml. shake culture vessels containing 100 ml. of medium; pH was determined immediately using a "Pye" model 79 pH meter.

The acids excreted by rhizobia growing in medium FB were identified and the amounts produced estimated. The carboxylic acids were estimated after separation in the gas liquid chromatograph (Rogosa & Love, 1968); pyruvate was determined colourimetrically (Friedemann & Haugen, 1943).

Results and Discussion.

Table 7 shows that all 20 strains of Rhizobium trifolii, cultured on Norris' (1965) medium, rapidly reduced the pH to below 6.0.

Growth of Rhizobium trifolii in medium FB with both NH_4Cl and $NaNO_3$ as sole nitrogen sources was accompanied by a rapid decrease in the pH of the medium (Table 8). It is to be noted, however, that the pH of the nitrate medium rose slightly in the first 24 hrs of growth.

These results suggested that organic acids were accumulating as a result of substrate utilization. Table 9 records the varying amounts of pyruvate and of the C_2 - C_4 carboxylic acids found in the acidified spent growth medium. Miss E. Donaldson, of this laboratory, using an ether extraction technique before separation of the acids in a gas liquid chromatograph, has shown that trace amounts of C_5 and C_6 carboxylic acids are also produced by many strains during growth in medium FB.

These results do not confirm the hypothesis of Norris (1965). Brockwell, Asuo & Rea (1966) reported acid production by Rhizobium isolates from Lotus spp., but considered their results to support Norris' (1965) hypothesis since there was a positive correlation between the final reaction of his medium and the effectiveness of the rhizobia. Jones & Burrows (1969) found a similar relationship for R. trifolii. It is considered that sufficient strains have not been investigated in this study to confirm any correlation between effectiveness and acid production. The final pH of Norris' (1965) medium did decrease with decreasing

TABLE 7.

Acid reaction of Norris' (1965) medium during growth of Rhizobium trifolii.

Strain	Indicator colour at (days) 3	7	28	Final pH
FA6	B‡	Y	Y	5.7
P3	Y	Y	Y	5.6
9CS	Y	Y	Y	5.3
1DL	Y	Y	Y	5.4
P1	B	Y	Y	5.3
1	B	Y	Y	5.6
P2	Y	Y	Y	5.4
8AL	Y	Y	Y	5.4
8	B	Y	Y	5.3
9CL	Y	Y	Y	5.6
7AL	B	Y	Y	5.5
6CL	Y	Y	Y	4.8
3AS	Y	Y	Y	5.3
7	Y	Y	Y	5.3
6	Y	Y	Y	5.1
8CS	Y	Y	Y	5.2
4AL	Y	Y	Y	5.0
7S	Y	Y	Y	5.2
W19	B	Y	Y	4.8
2	Y	Y	Y	5.5
Uninoculated control	B	B	B	6.1

‡ B = blue, Y = yellow

Strains are arranged in decreasing order of effectiveness.

TABLE 8.

Acid reaction of medium FB during growth of Rhizobium trifolii.

Time (hrs)	Strain	Nitrogen source: 0.02 M NH_4Cl	Nitrogen source: 0.02 M $NaNO_3$
0	P3	6.6	6.6
	FA6	6.6	6.6
	8CS	6.6	6.6
18	P3	6.5	6.8
	FA6	6.5	6.9
	8CS	6.6	6.9
24	P3	6.5	6.7
	FA6	6.2	6.9
	8CS	6.6	6.8
42	P3	5.7	5.9
	FA6	4.9	5.1
	8CS	5.4	5.8
48	P3	5.5	5.6
	FA6	4.9	5.1
	8CS	5.3	5.6
66	P3	5.0	5.1
	FA6	4.9	4.8
	8CS	4.9	4.9

TABLE 9.

Amounts (mM/l. spent medium) of acids produced by Rhizobium trifolii after 5 days' growth in medium FB.

Rhizobium strain	Acids produced					Final pH
	Acetic	Propionic	iso-Butyric	Butyric	Pyruvic	
FA6	1.4	1.5	T‡	0.9	0.07	5.1
P3	1.8	T	5.9	T	0.04	4.9
9CS	4.8	6.4	T	4.2	0.01	5.1
1DL	4.6	0.9	4.2	T	0.06	5.1
P1	T	0.6	1.0	-	0.04	5.3
1	1.5	T	8.9	-	0.21	5.0
P2	0.5	-	-	0.4	0.04	5.3
8AL	T	-	T	-	0.38	5.5
8	0.4	-	1.4	-	0.39	5.1
9CL	3.5	7.1	11.9	2.2	0.17	4.8
7AL	4.7	5.2	1.0	2.9	0.50	4.9
6CL	-	T	14.6	12.2	0.04	4.9
3AS	T	-	4.2	-	0.41	4.7
7	0.7	-	7.7	-	0.38	5.6
6	T	-	-	-	0.21	5.2
8CS	T	-	-	-	0.15	5.0
4AL	-	-	5.4	-	0.41	4.9
7S	0.8	-	0.9	-	0.27	4.9
W19	0.1	-	0.8	-	0.02	5.0
2	0.7	-	1.4	0.5	0.12	5.2

‡ T = trace

All amounts refer to a bacterial dry wt of 100 mg./l.

Strains are arranged in descending order of effectiveness.

effectiveness, though with one or two notable exceptions (Table 7). This is emphasized by the finding (Table 9) that more pyruvate was produced by strains which were less effective on S. 184 white clover than by those highly effective on this plant. Again, there were notable exceptions and the final pH's of the cultures appear independent of effectiveness. The contribution of pyruvate to the final pH of the medium cannot be assessed for two reasons. Firstly, the types and amounts of other acids produced varied markedly. Secondly, other acidic compounds may also be found in the spent medium. Mention has already been made of the traces of C_5 and C_6 carboxylic acids produced. In the NH_4Cl medium, utilization by rhizobia of the ammonium ion, resulting in chloride ion imbalance, would also be expected to cause a decrease in pH.

The early literature on Rhizobium contains a number of reports of acid production in pure culture. Anderson, Peterson & Fred (1928) found pyruvate in the spent growth medium of R. trifolii whilst Virtanen, Nordlund & Holland (1934) reported a butyric acid fermentation. The report of pyruvate production does not appear to have been further investigated while the long duration of the experiments of Virtanen et al. (1934), 306 hrs after a washing procedure, makes the interpretation of their data difficult. The results reported here confirm these early workers' findings. More recently, Damery & Alexander (1969) have reported amino-acid excretion by strains of R. meliloti.

Little is known of the pathways by which carboxylic acids and pyruvate are produced in rhizobia. Keele, Hamilton & Elkan (1969) have shown that glucose metabolism in Rhizobium japonicum

proceeds *via* the Entner-Doudoroff pathway, in which 2 moles of pyruvate are produced from 1 mole of glucose. Katznelson (1955) and Katznelson & Zagallo (1957) found most of the enzymes of Embden-Meyerhof-Parnas, the pentose-phosphate and the Entner-Doudoroff pathways in *R. phaseoli* and *R. meliloti*. However, Still & Wang (1964) consider that the Embden-Meyerhof-Parnas and the Entner-Doudoroff pathways do not operate simultaneously. The corresponding pathways in other rhizobia have not been determined.

Pyruvate is a key intermediate in the carbohydrate metabolism of bacteria. It is known to be involved in the production of poly-3-hydroxybutyrate, a carbon storage compound which can account for 50 p.c. of the dry weight of cells of *Rhizobium trifolii* (Forsyth, Hayward & Roberts, 1958; Vincent, Humphrey & North, 1962). Wilkinson (1963) has suggested that storage compounds are accumulated by bacteria whenever possible, but not at the expense of growth rate. Conditions suitable for the storage of poly-3-hydroxybutyrate prevail in medium FB. Side reactions may give rise to acetate (from acetyl-CoA) and butyrate (from 3-hydroxybutyryl-CoA) (Rose, 1968). Acetate may, alternatively, be derived from pyruvate *via* acetyl-P.

More difficult to explain is the production of propionic and *iso*-butyric acids. The pathway to propionate appears to involve the isomerization of succinyl-CoA to methylmalonyl-CoA (Wood & Utter, 1965), at least in the propionic acid bacteria. A similar route possibly occurs in the rhizobia though it may proceed *via* lactate, as in *Clostridium propionicum* (Rose, 1968).

Iso-butyrate is derived from valine in a Stickland reaction (Barker, 1961). However, it is not known if such a reaction,

mainly confined to Clostridium spp., occurs in rhizobia.

The derivation of these acids is at the moment a subject for speculation. The pathways to pyruvate, acetate and butyrate appear more certain than those to propionate and iso-butyrate. Nevertheless, the reasons for the excretion of these acids from the cell are not known.

Dudman & Heidelberger (1969) have shown pyruvate and acetate to be important constituents of the extracellular polysaccharide of Rhizobium trifolii. It is considered unlikely, however, that the methods employed in the determination of the acids would have removed such groups from the polysaccharide.

The most likely explanation for acid production by Rhizobium trifolii would appear to be a simple leak of these metabolic intermediates. It is possible that the high carbon content of the growth medium results in the organism obtaining most of its growth energy from substrate level phosphorylation, resulting in pyruvate accumulation within the cell. It is envisaged that the Krebs cycle, shown to exist in Rhizobium japonicum by Keele et al. (1969), is not fully functional until the carbon and energy source in the medium is limiting.

Acid production by bacteria is not unusual in vitro. It is, though, difficult to prove with soil bacteria that acids are excreted in vivo. The amounts of acids produced by rhizobia are extremely small and it is difficult, at the current time, to assess the importance of such small quantities in the soil.

EXPERIMENTAL WORK.

PART IV : THE EFFECT OF IRON ON THE GROWTH OF S. 184 WHITE CLOVER AND THE SURVIVAL OF RHIZOBIUM TRIFOLII IN ACID SOILS

Singer (1964) investigated the effect of Fe-EDTA on white clover growing in peat and brown earth soils. He found that, at pH 6.0 - 6.2, the aplication of iron-chelate produced a marked increase in the numbers of rhizobia in uninoculated brown earth. Survival of rhizobia in inoculated peat was also enhanced by Fe-EDTA treatment, but no effect was observed in the inoculated brown earth soil. The treatments did not affect clover yields.

Since high liming of acid hill soils is an uneconomic proposition, it was considered that this experiment should be repeated under more acid conditions. As low soil pH's are antagonistic to Rhizobium trifolii (van Schreven, 1958), all pots were inoculated.

At the same time, a comparison was made of $Ca(OH)_2$ and $Mg(OH)_2$ as liming agents to show differences in the effects of calcium, magnesium and pH on the symbiosis and on the free-living rhizobia.

Methods.

The pH of the brown earth soil, treated with potassium and phosphorus (as described in "Materials and Methods"), was raised to either 4.7 or 5.3 with either $Ca(OH)_2$ or $Mg(OH)_2$.

The treated soil was placed in glazed earthenware pots of 2.27 kg. capacity. After equilibration of the soil, the pots were inoculated with a mixture of effective rhizobia, strains P3,

Pl and 1DL, to give a final count of 400 rhizobia per g. of soil.

Twenty sterile seedlings per pot were sown, which, on establishment, were reduced by thinning to 10.

A concentrated solution of Fe-EDTA was diluted immediately prior to use to give 3 solutions containing 16, 32 and 160 ppm iron. Water was used as a control. For the first 5 weeks of the experiment, 100 ml. of each treatment were given weekly; for the rest of the growth period (17 weeks), the treatments were administered on alternate days. On the other days, the pots were watered if necessary.

The experiment was laid out in a random block design, 3 replicates in all.

Two croppings of clover were taken, the first after 12 weeks' growth, the second after a further 10 weeks'. The shoots were cut uniformly 10 mm. above soil level, dried at 105^{o} and their dry weights recorded.

At the end of the experiment, i.e. after the second cropping, the numbers of rhizobia per g. of soil were determined (Brockwell, 1963).

Results and Discussion.

The results (Tables 10 and 11; Appendices 3 and 4) show that the Fe_3-treatment caused a significant reduction in yield at both acid reactions, as did the Fe_2-treatment at the first cropping. Two explanations are available.

The poor yields could be due to the adverse effects of Fe-EDTA on nodulation (Lie & Brotonegoro, 1969). However, Gibson

TABLE 10.

The effect of Fe-EDTA on the growth of S. 184 white clover - first cropping.

Dry wt yield (g./pot), mean of 3 replicates

Liming agent	$Ca(OH)_2$		$Mg(OH)_2$		Means	
pH	4.7	5.3	4.7	5.3	4.7	5.3
Fe-treatment						
Fe_0	4.7	5.3	3.5	0.5	4.1	2.9
Fe_1	4.5	5.1	2.9	0.8	3.7	3.0
Fe_2	3.5	3.9	2.6	0.7	3.1	2.3
Fe_3	0.7	1.8	0.5	0.0	0.6	0.9
Means	3.4	4.0	2.4	0.5	2.9	2.3

Standard errors:				
	Block	± 0.12	pL	± 0.14
	pH	± 0.10	pF	± 0.20
	Lime	± 0.10	LF	± 0.20
	Fe	± 0.14	pLF	± 0.28

TABLE 11.

The effect of Fe-EDTA on the growth of S. 184 white clover - second cropping.

Dry wt yield (g./pot), mean of 3 replicates

Liming agent	$Ca(OH)_2$		$Mg(OH)_2$		Means	
pH	4.7	5.3	4.7	5.3	4.7	5.3
Fe-treatment						
Fe_0	9.0	9.6	6.3	2.3	7.7	6.0
Fe_1	9.6	8.2	6.6	2.9	8.1	5.6
Fe_2	8.8	8.1	5.6	2.0	7.2	5.1
Fe_3	4.1	3.1	0.4	0.2	2.3	1.7
Means	7.9	7.3	4.7	1.9	6.3	4.6

Standard errors: Block ± 0.25 pL ± 0.29
pH ± 0.20 pF ± 0.40
Lime ± 0.20 LF ± 0.40
Fe ± 0.29 pLF ± 0.57

(pers. comm.) has used Fe-EDTA as a source of iron in legume experiments for some years with no adverse effects on nodulation. Further, Lie & Brotonegoro's (1969) data suggest that nodulation should be inhibited at the low iron treatments, but reduced yields were not observed at these levels. Hewitt (1966) has suggested that the use of large amounts of a chelating agent may interfere in the specific requirements of a plant for other micronutrients.

The alternative explanation is one of iron toxicity. Little information is available on this subject. Demetriados (1938) showed that excess iron caused a number of toxic effects in a variety of plants, but not chlorosis. Somers, Gilbert & Shive (1942) found that soybeans grew free of pathological symptoms when the iron-manganese ratio in the substrate was between 1.5 : 1 and 2.5 : 1. In this range, the highest yields of respiratory CO_2 were obtained. It was subsequently shown that the symptoms of iron deficiency corresponded to those of manganese toxicity and vice versa (Somers & Shive, 1942).

Significant differences were also found between the yields of clover from calcium- and magnesium-treated soils (Tables 10 and 11). On the calcium-treated soils, better yields of clover were obtained in the first cropping at pH 5.3 than at pH 4.7 whilst the differences in yields of the second croppings at the two soil reactions were not significant. On the other hand, in the magnesium-treated soil better yields of clover were obtained from both croppings at pH 4.7. It is probable that the depression in clover growth at pH 5.3 results from excess magnesium inhibition of calcium uptake. Excess magnesium is also known to interfere with potassium

assimilation (Simpson, pers. comm.) which in turn affects the response of clover to phosphorus (van Schreven, 1958). However, this would seem an unlikely explanation in view of the treatment of the brown earth soil with potassium and phosphorus prior to potting.

The survival of Rhizobium trifolii in the soil was determined only at pH 4.7 since minimum fertilizer applications to hill areas are prescribed by economic considerations. It can be seen from Table 12 that, in spite of clover productivity being greater in the calcium-treated soil, better survival of rhizobia was found in the other soil at the two low iron treatments. This finding is of interest in view of the report of Norris (1958) that magnesium is an essential micronutrient for rhizobia.

These results, however, show that agronomically calcium is more important than magnesium and confirm previous data (Albrecht & Davis, 1929; Loneragan & Dowling, 1958; van Schreven, 1958) showing that the Rhizobium-Trifolium association suffers markedly if ample calcium is not available.

These findings can only be compared with those of Singer (1964) for the calcium-treated soil with the low iron treatments. It is to be noted that no improvement in survival of rhizobia was found between the Fe_0- and Fe_1-treatments at pH 4.7, the same result as Singer's (1964) at pH 6.0. He did not use the Fe_2-treatment which gave a much better survival of rhizobia at pH 4.7.

The feasibility of such iron treatments in the field is doubtful. It would appear uneconomic to treat vast areas of hill land as frequently as the pots were treated in this experiment. If, however, it could be shown that one large Fe-EDTA treatment could

TABLE 12.

Counts of *Rhizobium trifolii* ($x10^{-1}$)/g. soil after 22 weeks' growth of Fe-treated S. 184 white clover at pH 4.7.

Liming agent	$Ca(OH)_2$	$Mg(OH)_2$
Fe-treatment		
Fe_0	56.3	28.2 x 10
Fe_1	28.3	70.6 x 10^2
Fe_2	35.3 x 10^2	41.0 x 10^2

95 p.c. confidence limits corresponding to counts above.

Fe_0	21.9 - 146.0	(16.9 - 73.0) x 10
Fe_1	10.5 - 76.1	(27.1 - 184.2) x 10^2
Fe_2	(13.1 - 954) x 10^2	(1.6 - 10.6) x 10^2

Soil inoculated with approximately 400 rhizobia/g.

improve survival of rhizobia, it would be possible to give such a treatment along with other intermittent fertilizer applications in the maintenance of hill pastures.

EXPERIMENTAL WORK.

PART V : THE EFFECTS OF MANGANESE ON THE RHIZOBIUM-TRIFOLIUM SYMBIOSIS

a. Growth of Rhizobium trifolii in the presence of manganese.

The fact that most metals become more available in soil as pH decreases (Truog, 1946; Lucas & Davis, 1961) suggests the possibility that metal antagonism to bacterial growth may be a factor in the small numbers of Rhizobium trifolii found in acid hill soils.

A study was therefore made of the effects of various metals on the growth of Rhizobium trifolii.

Methods.

The metals investigated for their effects on the growth of Rhizobium trifolii were iron (as Fe-EDTA), manganese (as $MnSO_4.4H_2O$), copper (as $CuSO_4.5H_2O$), zinc (as $ZnSO_4.7H_2O$), magnesium (as $MgSO_4.7H_2O$), boron (as H_3BO_3), cobalt (as $CoSO_4.7H_2O$) and molybdenum (as $Na_2MoO_4.2H_2O$). Concentrated solutions of these salts were prepared, autoclaved and aseptically added to medium FB (pH 5.7) prior to sloping in the concentrations detailed in Table 13.

Two drops of a standard suspension of a 2-day culture were run down each slope. The cultures were put to grow at 27^{o}.

Results and Discussion.

The results (Table 13) show that the strains varied markedly in their sensitivity to the metals but there appears to be little difference between the effective and ineffective strains.

TABLE 13 a.

Growth of Rhizobium trifolii in presence of various metals.

Metal	Concentration (mM)	Rhizobium strain: Effective P3	P1	8	Ineffective 2	6CL	3AS	8CS
Fe	0.1	+++	++	++	++	+	++	++
	0.5	+++	++	++	++	++	++	++
	1.0	+++	++	++	++	++	++	++
Mn	4.0	+++	++	+++	++	+	++	+++
	8.0	+++	+	+++	++	+	++	+++
	16.0	++	+	++	+	+/-	+	+
Cu	0.2	+++	++	+++	++	++	+++	+++
	0.4	+++	++	++	++	+	++	++
	0.8	-	-	-	-	-	-	-
Zn	0.2	+++	++	++	++	++	+++	+++
	0.4	+++	++	++	++	++	++	+++
	0.8	+++	+	+	++	++	++	++

Growth estimated visually after 10 days.

- no growth
+ slight growth
+/- growth barely visible
++ confluent growth
+++ heavy confluent growth

TABLE 13b.

Growth of Rhizobium trifolii in presence of various metals.

Metal	Concentration (mM)	Rhizobium strain: Effective P3	Effective P1	Effective 8	Ineffective 2	Ineffective 6CL	Ineffective 3AS	Ineffective 8CS
Mg	4.0	+++	++	+	++	+	++	++
	8.0	+++	+	++	+	+	+	++
	16.0	+++	+	++	+	++	+	++
B	4.0	+++	++	++	++	+	++	++
	8.0	+++	++	++	++	+	++	++
	16.0	++	+	++	++	+	++	+
Co	0.05	+++	++	++	++	+	++	+++
	0.50	+++	++	++	++	+	+	++
Mo	0.1	+++	++	++	+	+	++	++
	0.5	++	++	++	+	+	++	+
	1.0	++	+	+	++	+	+	+

Growth estimated visually after 10 days.

- no growth
+/- growth barely visible
+ slight growth
++ confluent growth
+++ heavy confluent growth

The possible exception is 16 mM manganese on which the ineffective strains had grown only slightly after 10 days whereas 2 of the 3 effective strains showed confluent growth. Inhibition of growth was also found at the higher concentrations of copper and molybdenum.

Of these metals, it is generally considered that manganese toxicity is a problem in acid hill soils. Molybdenum availability decreases with pH (Lucas & Davis, 1961) whilst copper, though readily available, is not present in large amounts. Manganese, however, does occur in high concentrations in the acid hill soils of the Scottish Southern Uplands (Muir, 1956).

Further investigations of the growth of Rhizobium trifolii were undertaken. The determination of the growth rates of the various strains in the presence of manganese proved unsuccessful by turbidometric methods. A small increase in nephelometer readings was usually obtained but, after about 2 hrs, the rate of increase slowed and the readings became scattered. Microscopic examination of the cultures showed the rhizobia to be extremely pleomorphic as compared with controls.

Investigation of this phenomenon, using Bacillus and Pseudomonas spp. from the Edinburgh collection, showed that manganese had a similar morphological effect on these organisms. They, however, were more sensitive than the rhizobia to manganese.

The effect of manganese on all the micro-organisms was found to be alleviated by the addition of magnesium to the medium. However, even at magnesium concentrations equimolar with those of manganese the morphology of the bacteria differed slightly from that of the controls.

The changes in morphology associated with high manganese concentrations were, then, the cause of the irregular growth curves. Growth rates were subsequently obtained from dry weight determinations. The results (Table 14) show a marked reduction in the growth rate of all strains in the presence of 4 mM (225 ppm) manganese. This corresponds well with a decrease in respiration rate, measured with the Clark electrode, though in one or two cases there was little difference in the respiration rates of the treated and control rhizobia (Table 15).

The finding that rhizobia growing on manganese-rich media tended to regain their normal morphology when magnesium was present indicates that manganese induces magnesium deficiency. Vincent & Colburn (1961) reported that magnesium-deficient rhizobia were of variable morphology. Previously Webb (1949) had shown magnesium deficiency to cause abnormal cell development in the Gram positive spore-formers and Kennell (1967) and his group have reported the same phenomenon in Aerobacter aerogenes.

Growth in a nutritionally unbalanced environment presents difficulties to any organism. That rhizobia and agrobacteria (Clark, 1969) can grow in the presence of 900 ppm manganese suggests that these organisms possess some property not found in many other bacteria. Manasse & Corpe (1967) have shown that the cell wall of Agrobacterium tumefaciens contains relatively large amounts of aspartic acid. They suggest that this reflects a high level of intermolecular bonding, possibly between the mucopeptide and a protein granule layer, resulting in added stability of the cell

TABLE 14.

Doubling times of *Rhizobium trifolii* strains in medium FB and medium FB + 200 ppm manganese.

Rhizobium strain	Doubling time (hrs)	
	Medium FB	Medium FB + 200 ppm Mn
FA6	4.3	9.6
P3	2.0	5.1
9CS	2.4	4.5
1DL	4.9	6.3
P1	3.5	7.1
1	2.7	6.4
P2	2.1	6.1
8AL	4.3	9.2
8	3.9	4.3
9CL	4.1	9.3
7AL	3.1	8.0
6CL	4.7	9.7
3AS	3.4	6.0
7	2.9	6.2
6	3.1	6.8
8CS	3.6	6.8
4AL	3.1	7.0
7S	3.4	4.2
W19	3.3	6.9
2	4.6	8.0

TABLE 15.

Respiration rates of Rhizobium trifolii in medium FB and medium FB + 200 ppm manganese.

Rhizobium strain	Respiration rate (p.c./min.)	
	Medium FB	Medium FB + 200 ppm Mn
FA6	1.0	1.1
P3	2.1	1.0
9CS	0.6	1.5
1DL	1.7	1.6
P1	1.8	1.1
1	3.8	1.9
P2	1.1	1.5
8AL	2.6	1.5
8	1.0	0.6
9CL	4.0	1.8
7AL	2.6	1.4
6CL	2.7	1.4
3AS	2.4	1.8
7	1.9	0.9
6	3.5	2.0
8CS	2.1	1.3
4AL	3.4	2.1
7S	2.5	2.3
W19	2.2	1.0
2	0.9	1.0

envelope. In view of the close relationship between Agrobacterium and Rhizobium it is probable that a similar cell wall structure is to be found in rhizobia. Such a cell wall might explain the ability of these micro-organisms to survive the unbalanced conditions which other bacteria, owing to a weaker cell wall matrix, cannot tolerate.

Manganese and magnesium, though chemically dissimilar, are known to be alternative co-factors for many enzyme systems (Dixon & Webb, 1964). For these systems, with a concentration of magnesium sufficient to produce maximal activation, addition of manganese causes inhibition. Abelson & Aldous (1950) reported that low levels of magnesium predispose micro-organisms to the toxic effects of other divalent cations, though Webb (1968) reports that under these conditions manganese substitutes for magnesium in ribosomes, leaving free magnesium for other metabolic purposes.

The reversal by magnesium of the effect of excess manganese on the morphology of Rhizobium trifolii suggests that competition for enzyme sites occurs within the organism under excess manganese conditions and this results in the inhibition of some enzyme systems; the resultant imbalance manifests itself in the morphological changes described. It would seem unlikely that minimal magnesium levels are only predisposing to manganese toxicity, as suggested by Abelson & Aldous (1950), since equimolar amounts of magnesium are required to alleviate the manganese toxicity symptoms.

b. The effect of manganese on the effectiveness of Rhizobium trifolii.

It is known that unbalanced growth conditions can change the effectiveness of rhizobia. Reid (1940) and Badawy & Allen (1963) have shown that calcium deficiency can reduce effectiveness in rhizobia, while Holding, Tilo & Allen (1960) found the same phenomenon in rhizobia grown on amino-acid-rich media. It was therefore considered highly possible that the unbalanced growth conditions encountered in manganese-rich media might similarly modify the effectiveness of rhizobia. Less certain was whether any attenuated strain produced would subsequently revert to normalcy.

Methods.

Ten strains of Rhizobium trifolii were subcultured on medium FB containing 16 mM $MnSO_4.4H_2O$ (900 ppm manganese) at fortnightly intervals for 5 months. Controls were concurrently cultured on medium FB without added manganese. All strains were then inoculated over the roots of S. 184 seedlings on nitrogen-free agar slopes, which were arranged in a pure random design (6 replicates). Wet weights were, as usual, recorded after 10 weeks' growth.

The manganese-treated and control organisms were then subcultured on medium FB without added manganese for a further 5 months and tested for efficiency of symbiosis as before.

A second experiment, differing from the first only in that the rhizobia were subcultured on the various media once in each part and that the plants were arranged in a random block design with 6 replicates, was also performed.

Results and Discussion.

Table 16 (along with Appendix 5) shows that the responses of the white clover plants nodulated by manganese-treated effective strains of Rhizobium trifolii were very highly significantly smaller than those of the control plants. The only exceptions were those of the plants nodulated by strain P3R, the rough derivative of strain P3.

From Table 17 (and Appendix 6), it can be seen that after the attenuated rhizobia had been cultured on medium FB, 3 of the 5 strains, which had previously caused a reduced growth response in S. 184 clover, had regained their effectiveness. Nevertheless, the overall response from the clover nodulated by the attenuated strains was still very highly significantly lower than that of the controls, owing to the strains 8AL and P3 which had not regained their former effectiveness.

Demerec & Hanson (1951) reported on the mutagenic action of manganese(II) ions in bacteria. Sarachek (1960) showed that manganese(II) ions were also mutagenic to yeasts. It would, however, seem doubtful that manganese was exerting a mutagenic effect in this experiment since Demerec & Hanson (1951) showed that mutation only occurred in bacteria which had been pre-treated in liquid culture. This has subsequently been confirmed in other laboratories (MacPhee, pers. comm.) though these reports do not preclude the possibility that manganese can cause mutagenesis in solid culture.

The finding that, on return to balanced media, the attenuated rhizobia tended to regain their former effectiveness, suggests that the reduction in effectiveness is a phenotypic change,

TABLE 16.

The response of S. 184 white clover to manganese-treated and untreated rhizobia.

Plant yield (wet wt) mg./tube of 3 plants (mean of 6 replicates)

	Rhizobium strain	Mn-treated		Untreated
			SE: ±38	
Effective	P3	154		452
	P3S	235		455
	P3R	468		471
	8AL	69		438
	9CS	212		351
	8	96		280
Ineffective	6CL	228		211
	3AS	144		191
	7S	59		109
	2	62		48
	Mean	173	SE: ±12	300

TABLE 17.

The response of S. 184 white clover to manganese-treated and untreated rhizobia after a further 5 months' growth on medium FB.

Plant yield (wet wt) mg./tube of 3 plants (mean of 6 replicates)

	Rhizobium strain	Mn-treated		Untreated
			SE: ±19	
Effective	P3	282		465
	P3S	424		383
	P3R	378		400
	8AL	390		444
	9CL	581		583
	8	373		367
Ineffective	6CL	268		310
	3AS	272		294
	7S	177		166
	2	70		82
Mean		322	SE: ±6	350

as was the change in morphology described in the previous section. Orgel & Orgel (1965) reported that all complexes of manganese(II) with ligands containing oxygen and nitrogen are isomorphous with the corresponding magnesium complexes, even though the ionic radius of manganese(II) is somewhat larger than that of magnesium ions.

It is, therefore, possible that in important enzymes associated with the nitrogen-fixing system magnesium has been replaced by manganese. This would give enzyme complexes which were stereochemically normal but the specifity of which was affected. On return to balanced media, the rhizobia tended to regain their former cation balance.

It is considered that the results of the second (1 subculture) experiment support the suggestion that manganese acted primarily on the phenotype of the bacterium. Tables 18 and 19 (Appendices 7 and 8) show that, during 2 weeks' growth on the manganese-rich medium and a further 2 weeks' on medium FB, the rhizobia did not change in their efficiency in nitrogen fixation.

It would therefore be of interest to ascertain, firstly, whether growth of effective Rhizobium strains on a manganese-magnesium-rich medium would result in reduced efficiency of symbiosis; and secondly, whether growth of the laboratory-attenuated strains on a medium rich in magnesium would hasten their return to full effectiveness.

TABLE 18.

The response of S. 184 white clover to manganese-treated and untreated rhizobia (1 subculture).

Plant yield (wet wt) mg./tube of 3 plants (mean of 6 replicates)

	Rhizobium strain	Mn-treated		Untreated
			SE: ± 34	
Effective	P3	500		481
	P3S	442		393
	P3R	485		498
	8AL	217		231
	9CS	414		372
	8	219		243
Ineffective	6CL	107		106
	3AS	167		124
	7S	76		86
	2	63		62
Mean		269	SE: ± 24	260

TABLE 19.

The response of S. 184 white clover to manganese-treated and untreated rhizobia (1 subculture) after a further 2 weeks' growth on medium FB.

Plant yield (wet wt) mg./tube of 3 plants (mean of 6 replicates)

	Rhizobium strain	Mn-treated		Untreated
			SE: ±32	
Effective	P3	383		413
	P3S	443		443
	P3R	451		429
	8AL	340		381
	9CL	370		400
	8	249		230
Ineffective	6CL	77		109
	3AS	171		160
	7S	89		83
	2	64		69
Mean		264	SE: ±10	272

c. The effect of manganese on the establishment of clover.

Somers & Shive (1942) reported that the symptoms of manganese toxicity in plants corresponded with those of iron deficiency. Vose & Jones (1963) found clear varietal differences between four strains of white clover in the degree of manganese toxicity symptoms. They also reported that the number of nodules on white clover was reduced in the presence of manganese, suggesting at least two ways in which manganese might affect clover establishment *in vivo*.

However, no data are available to show the manner in which manganese reduces nodule numbers. It was therefore considered that an investigation of the effects of manganese on S. 184 white clover should be undertaken.

i. The effect of manganese on clover growth.

In the two pot experiments designed to study the effects of manganese on S. 184 clover growth, the pots were arranged in random block designs. However, the plants inoculated with effective strains of *Rhizobium trifolii* were placed on one side of the greenhouse, those inoculated with ineffective strains on the other. This layout was adopted to reduce the contamination of ineffective treatments by effective rhizobia since Singer, Holding & King (1964) reported that, in brown earth soils, a small proportion of effective rhizobia was sufficient to produce a highly effective response.

In the two experiments, the effective and ineffective treatments were therefore separately analysed. For comparison purposes, the results were subtracted from each other (effective minus ineffective) and a further analysis performed (Pearce, 1953).

Experiment 1 - Watering experiment.

Methods.

Prepared soil of pH 4.5 or 5.0 (see "Materials and Methods") was equilibrated in glazed earthenware pots (178 x 127 mm.) of 2.27 kg. capacity. Each pot was inoculated with one of the strains of Rhizobium trifolii selected (7AL and 3AS - ineffective; P3 and 1DL - effective). Twenty sterile seedlings were then sown in each pot; on establishment, these were reduced to 10 by thinning.

The pots were treated weekly for 5 weeks and then alternate days for the rest of the experiment (12 weeks) with 100 ml. of a solution containing either 0, 10 or 25 ppm manganese as $MnSO_4.4H_2O$ or 10 ppm iron and manganese as Fe-EDTA and $MnSO_4.4H_2O$. This last treatment was to test whether iron would alleviate any manganese toxicity symptoms that might develop. On other days, the moisture regime was kept constant with tap water.

At the end of the growth period, the clover shoots, cut 10 mm. above soil surface, were harvested and dry weights determined. The survival of rhizobia in the low manganese treatments was also determined by the method of Brockwell (1963).

Results and Discussion.

The yields obtained (Tables 20 and 21; Appendices 9 and 10) confirm the importance of liming. For both the effectively and ineffectively nodulated plants, the pH factor is very highly significant; the comparison of effective and ineffective strains (Appendix 11) also shows liming to have a significant effect.

TABLE 20.

The effect of manganese on the yield of S. 184 white clover growing in brown earth soil - watering experiment.

Dry wt yield (g./pot), mean of 3 replicates

	Ineffective strains					
	7AL		3AS		Means	
pH	4.5	5.0	4.5	5.0	4.5	5.0
Mn-treatment						
Mn_0	1.7	3.0	2.3	4.0	2.0	3.5
Mn_{10}	2.1	4.9	3.6	4.9	2.9	4.9
Mn_{25}	1.7	3.6	4.3	4.8	3.0	4.2
Fe_{10}/Mn_{10}	2.8	4.3	3.0	4.7	2.9	4.5
Means	2.1	3.9	3.3	4.6	2.7	4.3

Standard errors:				
Block	± 0.27	pR	± 0.31
pH	± 0.22	pM	± 0.44
Rhizobia	± 0.22	RM	± 0.44
Manganese	± 0.31	pRM	± 0.62

TABLE 21.

The effect of manganese on the yield of S. 184 white clover growing in brown earth soil - watering experiment.

Dry wt yield (g./pot), mean of 3 replicates

	Effective strains					
	P3		1DL		Means	
pH	4.5	5.0	4.5	5.0	4.5	5.0
Mn-treatment						
Mn_0	4.4	7.5	4.3	9.1	4.4	8.3
Mn_{10}	6.3	8.4	5.2	8.6	5.8	8.5
Mn_{25}	7.3	8.1	6.2	9.1	6.8	8.6
Fe_{10}/Mn_{10}	5.8	8.1	5.3	8.3	5.6	8.2
Means	6.0	8.0	5.3	8.8	5.7	8.4

Standard errors:				
Block	± 0.31	pR	± 0.36
pH	± 0.25	pM	± 0.51
Rhizobia	± 0.25	RM	± 0.51
Manganese	± 0.36	pRM	± 0.71

No reduction in yield as a result of manganese was recorded. This suggests that manganese toxicity may be a more important problem during the early stages of growth, possibly during nodulation, when only low concentrations of manganese were present in the treated soils.

Table 22 records the survival of rhizobia in the Mn_0 and Mn_{10} watering treatments. It can be seen that liming the soil to pH 5.0 provided conditions far more favourable for the growth and survival of the rhizobia than did the lesser lime treatment. The 4 strains varied in growth characteristics but it is to be noted that better survival was observed in the Mn_{10} treatment. This corresponds to the increased clover yield observed with this treatment, presumably associated with a greater rhizosphere stimulation, but no reliable explanation can be offered.

Experiment 2 - Incorporation experiment.

Methods.

Brown earth soil was prepared and limed to pH 4.5 or 5.0 and equilibrated as described in "Materials and Methods". Four levels of manganese, as $MnSO_4.4H_2O$, were incorporated, the final "exchangeable" contents being 5.7, 10.1, 20.0 and 64.1 ppm.

The use of 127 mm. plastic pots allowed a 5 block design. Each pot held 1.4 kg. wet soil. Two effective strains (FA6 and 9CS) and 2 ineffective strains (7 and W19) were each inoculated into the soil at the various manganese levels to give counts of approximately 400 rhizobia per g. wet soil.

TABLE 22.

Counts of rhizobia ($\times 10^{-1}$)/g. soil from manganese watering experiment after 17 weeks' growth of S. 184 clover.

	Mn-treatments			
	Mn_0		Mn_{10}	
pH	4.5	5.0	4.5	5.0
Rhizobium strain				
P3	8.7	410	81	113
1DL	4.0	56.5	11.3	380
7AL	3.5	56.5	56.5	182
3AS	4.0	188	410	5440
95 p.c. confidence limits corresponding to counts above				
P3	3.3 - 23.0	160 - 1060	31 - 212	44 - 292
1DL	1.2 - 12.8	21.9 - 146	4.4 - 29.2	150 - 980
7AL	1.1 - 11.9	21.9 - 146	21.9 - 146	69 - 484
3AS	12 - 12.8	72 - 490	160 - 1060	2060 - 14380

Soil inoculated with approximately 400 rhizobia/g.

Fifteen sterile seedlings were then sown in each pot. On establishment, this number was reduced to 8 by thinning. Dry weights of shoots, cut 10 mm. above soil surface, and number of rhizobia surviving in the soil (Brockwell, 1963) were determined after 4 months.

Results and Discussion.

The results (Tables 23 and 24; Appendices 12 and 13) show a very highly significant reduction in the yields of the effectively nodulated clover as soil manganese content increased. The response of the ineffectively nodulated plants was not correlated with the soil manganese; this is to be expected in view of the poor growth response of S. 184 clover to these strains.

A very highly significant effect due to manganese was also found in the comparison of effective and ineffective performance (Appendix 14). This was entirely due to the performance of the effective strains, to which a very marked reduction in response by S. 184 clover occurred at 10.1 ppm soil manganese as compared with the 5.7 ppm level.

Significant pH-strain and pH-manganese interactions were also found for the effective strains. These derive from the different response characteristics of S. 184 clover to nodulation by the 2 strains. Strain 9CS induced a greater response at pH 4.5 whilst at pH 5.0, strain FA6, an isolate from improved hill soil, was the better performer.

Similarly, the pH-manganese interaction is explained by differences in total yields at different lime and manganese levels.

TABLE 23.

The effect of manganese on the yield of S. 184 white clover growing in brown earth soil - incorporation experiment.

Dry wt yield (g./pot), mean of 5 replicates

	Ineffective strains					
	7		W19		Means	
pH	4.5	5.0	4.5	5.0	4.5	5.0
Mn concentration (ppm)						
5.7	3.6	5.1	4.3	4.6	4.0	4.9
10.1	5.0	3.9	3.7	4.1	4.4	4.0
20.0	5.5	4.4	4.0	4.9	4.8	4.7
64.1	3.0	3.7	4.1	3.9	3.5	3.8
Means	4.3	4.3	4.0	4.4	4.2	4.4

Standard errors:				
	Block	± 0.41	pR	± 0.37
	pH	± 0.26	pM	± 0.52
	Rhizobia	± 0.26	RM	± 0.52
	Manganese	± 0.37	pRM	± 0.73

TABLE 24.

The effect of manganese on the yield of S. 184 white clover growing in brown earth soil - incorporation experiment.

Dry wt yield (g./pot), mean of 5 replicates

	Effective strains					
	FA6		9CS		Means	
pH	4.5	5.0	4.5	5.0	4.5	5.0
Mn concentration (ppm)						
5.7	9.0	8.8	9.5	7.9	9.3	8.4
10.1	3.9	4.3	5.0	5.1	4.5	4.7
20.0	3.3	4.3	3.9	3.7	3.6	4.0
64.1	2.6	2.8	2.0	1.9	2.3	2.4
Means	4.7	5.1	5.1	4.7	4.9	4.9

Standard errors:		
Block	± 0.21	pR ± 0.19
pH	± 0.14	pM ± 0.27
Rhizobia	± 0.14	RM ± 0.27
Manganese	± 0.19	pRM ± 0.38

Table 25 records the numbers of rhizobia per g. wet soil. It is to be noted that at pH 4.5 no differences were found in the numbers of rhizobia surviving at the various manganese levels. At pH 5.0, however, all counts declined between the 5.7 ppm and 10.1 ppm manganese treatments irrespective of the plant response characteristics noted above. No differences were found in the counts at 10.1 ppm and 20.0 ppm manganese. It is also of interest that only in the untreated soil at pH 5.0 was an increase in numbers of rhizobia recorded.

It is, however, difficult to assess the contributions to the poor survival of these bacteria in the soil made by their longer doubling times in the presence of manganese (Part V, section a), their acid sensitivity (van Schreven, 1958) and their natural decline with time outside the legume rhizosphere (Nutman, 1963).

TABLE 25.

Counts of rhizobia (x 10^{-1})/g. soil from manganese incorporation experiment after 17 weeks' growth of S. 184 clover.

	Mn levels (ppm)					
	5.7		10.1		20.0	
pH	4.5	5.0	4.5	5.0	4.5	5.0
Rhizobium strain						
FA6	11.3	410	24.2	56.5	7.2	14.1
9CS	11.4	282	10.8	36.4	5.5	19.6
7	3.0	126	5.5	10.8	1.0	10.8
W19	8.0	290	7.2	28.3	4.9	11.3

95 p.c. confidence limits corresponding to counts above

FA6	4.4 - 29.2		9.0 - 64.9		2.7 - 19.6	
	160 - 1060		21.9 - 146.0		5.4 - 36.6	
9CS	4.4 - 29.5		4.2 - 28.1		1.9 - 16.0	
	109 - 730		13.7 - 96.8		7.4 - 51.9	
7	0.9 - 10.6		1.9 - 16.0		0.1 - 7.7	
	47 - 338		4.2 - 28.1		4.2 - 28.1	
W19	3.0 - 21.5		2.7 - 19.6		1.6 - 14.6	
	110 - 730		10.5 - 76.1		4.4 - 29.2	

Soil inoculated with approximately 400 rhizobia/g.

ii. The effect of manganese on nodulation.

Whilst it is known that manganese causes a reduction in nodule numbers of beans (Dobereiner, 1966) and white clover (Vose & Jones, 1963), no data are available to indicate how manganese might exert its effect. Low numbers of rhizobia, which in acid soils must be accounted part of the problem of clover establishment (Jones, 1966), were not a factor in these experiments.

It is therefore more likely that the low nodule counts derived from an effect of manganese on the nodulation process. The interdependence of Rhizobium and host is most sensitive to interference during the early stages of nodulation. Experiments were therefore designed to confirm that manganese does reduce nodule numbers on white clover and to show whether its presence delays the onset of nodule appearance and reduces the rate of nodulation.

Experiment 1 - Agar experiment.

Methods.

Four levels of manganese (3, 13, 28 and 53 ppm), as $MnSO_4.4H_2O$, were added to nitrogen-free agar immediately prior to sloping.

After establishment of 3 sterile S. 184 seedlings, each tube was inoculated with 1 ml. of a test suspension of Rhizobium trifolii. Tubes were arranged in a random block design, 6 blocks, and were inspected at 3 day intervals for 3 weeks and at the time of harvesting (70 days) for nodulation; nodule numbers were determined on each occasion.

After 10 weeks' growth, the plants were harvested and fresh weights determined.

Results.

Table 26 (see also Appendix 15) records the yields of S. 184 clover at the different manganese levels. At 10 ppm additional manganese, the 7 most effective Rhizobium strains induced an average clover response 48 p.c. lower than that of the 3 ppm control. This trend continued at 28 ppm manganese whilst at 53 ppm manganese little difference in the yields of clover nodulated by different strains was found.

The importance of strain variation can be seen from these results. With, for example, strain 1 the largest reduction in yield occurred between 3 and 13 ppm manganese, whilst with strain P3 the largest reduction in yield was found between 13 and 28 ppm.

Three days after inoculation of the plant cultures, very few nodules had appeared. Similarly, throughout the whole of the growth period, nodules did not form on the roots of clover growing in the presence of 53 ppm manganese, except in one or two isolated instances. The third day and the 53 ppm manganese counts were therefore omitted from the statistical analysis. Further, as a large number of the counts remaining were zero, the analysis was performed not on the numbers of nodules per tube of three plants but on a square root transformation of each result, $x' = (x + 0.5)^{0.5}$ where x = number of nodules per tube.

A table of the results as analysed is to be found in Appendix 16. In Figure 5, the effect of increased manganese levels

TABLE 26.

The effect of manganese on the yield of S. 184 white clover growing on nitrogen-free mineral salts agar.

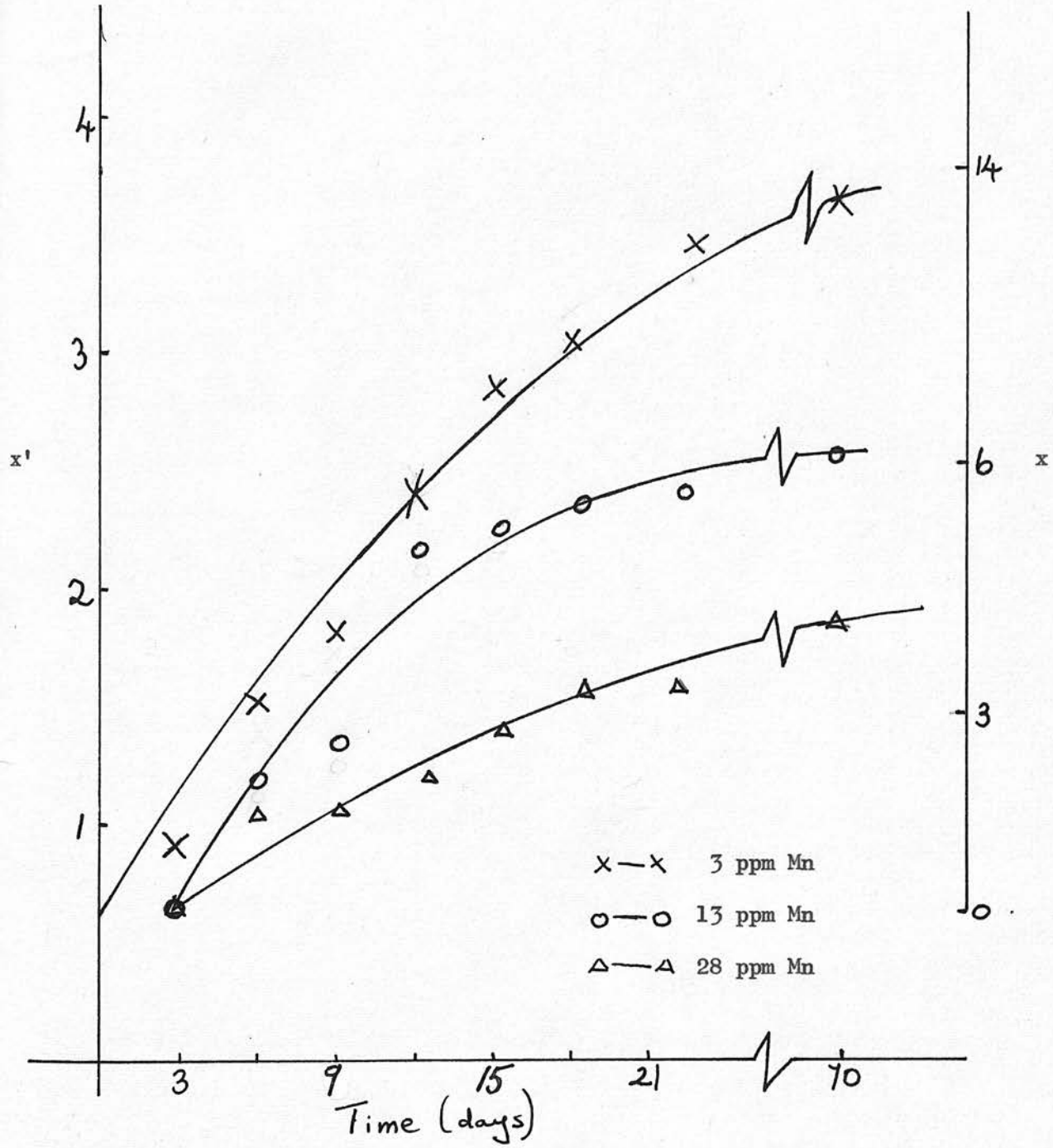
Plant yield (wet wt) mg./tube of 3 plants (mean of 6 replicates)

Rhizobium strain	Mn concentration (ppm)			
	3	13	28	53
		SE: ± 37		
1	528	232	78	67
P3	374	260	97	68
1DL	339	132	108	50
P1	282	137	114	48
4AL	174	94	82	51
7AL	121	80	82	48
6	117	82	74	40
7	110	157	119	37
2	96	121	77	52
9CL	88	98	94	60
Means: SE: ± 12	223	139	93	52

FIGURE 5.

The effect of manganese on nodulation by Rhizobium trifolii strain 1DL.

Plot of Time against x' where $x' = (x + 0.5)^{0.5}$ and x = no. of nodules/tube of 3 plants.



on the nodulation of clover by strain 1DL is shown graphically. It can clearly be seen that nodulation, as recognized by nodule appearance, started earlier at the 3 ppm manganese level than at the other levels. It can also be seen that nodulation proceeded faster at 3 ppm manganese than at 13 ppm, which itself was less inhibitory to nodulation than 28 ppm of the metal.

The results for strain 1DL are typical of 6 of the 10 strains examined, whether effective or ineffective in nitrogen fixation, in that 13 ppm manganese both delayed the development of the nodule and reduced nodule numbers. The effect of additional manganese was more variable, further delaying and reducing nodulation by some strains but not by others.

Nodulation by strains P1, 6 and 7 was not reduced at 13 ppm manganese as compared with 3 ppm but, in the case of strain P1, the response of S. 184 clover was significantly smaller than that at the control level.

Nodulation by strain 9CL was not affected by manganese (except at the 53 ppm level) and this is reflected in the similar yields at different manganese concentrations of clover nodulated by this strain (Table 26).

Experiment 2 - Pot experiment.

Methods.

Before potting, amended brown earth, pH 5.0, (see "Materials and Methods") was treated with $MnSO_4.4H_2O$ to give final levels of 5.7, 32.2, 85.6 and 125 ppm "exchangeable" manganese. The

127 mm. plastic pots were arranged in 2 random blocks, the ineffective strains, 7 and W19, on one side of the glasshouse, the effective strains, FA6 and 9CS, on the other. On establishment, the clover plants were reduced by thinning from 10 to 5.

After 21, 25, 29, 33, 41 and 112 days, plants from each soil-strain treatment were examined for nodules, after carefully washing the roots free of soil particles.

Results.

Nodules were at no time found on clover growing at the 125 ppm manganese level, and the results for this manganese concentration were therefore omitted from the analysis.

Tables 27 and 28 (along with Appendices 18 and 19) show a reduction in both ineffective and effective nodule numbers as nutrient manganese content increased. The results therefore confirm those of the agar experiment in showing inhibition of nodulation due to manganese.

For comparison purposes, the results were treated according to the method of Pearce (1953). Appendix 20, however, shows no significant manganese effect in the comparison of the two strain types. This is considered to reflect small differences in the nodulation patterns of the individual strains, rather than gross effective-ineffective differences.

Discussion.

Dixon (1969) has reviewed current knowledge of the nodulation process in legumes. The first sign of nodulation is the

TABLE 27.

The effect of manganese on the number of nodules developing on the roots of S. 184 white clover growing in brown earth soil (pH 5.0).

Numbers of nodules/plant (mean of 10 plants)

	Ineffective strains					
	7			W19		
Mn concentration (ppm)	5.7	39.2	85.6	5.7	39.2	85.6
Time (days)						
21	1.7	0.0	0.7	1.7	1.0	1.8
25	1.6	0.2	0.5	1.6	0.7	0.9
29	2.0	0.6	0.6	1.8	0.8	0.8
33	2.3	1.2	0.9	2.0	1.0	1.0
37	3.1	1.4	1.0	2.4	1.4	1.0
41	3.4	1.6	1.0	2.8	1.5	0.9
112	3.4	1.8	1.2	3.4	1.8	1.3

TABLE 28.

The effect of manganese on the number of nodules developing on the roots of S. 184 white clover growing in brown earth soil (pH 5.0).

Numbers of nodules/plant (mean of 10 plants)

	Effective strains					
	FA6			9CS		
Mn concentration (ppm)	5.7	39.2	85.6	5.7	39.2	85.6
Time (days)						
21	1.2	1.5	0.0	0.8	0.7	0.1
25	1.6	0.7	0.1	1.1	0.6	0.4
29	1.7	0.7	0.4	2.1	0.9	0.6
33	1.8	1.1	0.4	2.3	1.0	0.8
37	2.0	1.4	0.3	3.3	1.5	1.1
41	3.0	1.9	0.7	3.1	1.7	1.3
112	4.1	2.5	1.1	3.7	2.7	2.0

curling of the legume root hairs, though this is not an invariable indication (Nutman, 1959). Deformation of the root hairs has been attributed to the action of indole-3-acetic acid (IAA), formed by the Rhizobium from tryptophan excreted by the plant. Sahlman & Fahraeus (1962), however, consider it unlikely that IAA is the sole agent responsible since the reaction of root hair curling is more specific than could be obtained with IAA alone. The importance of this suggestion is emphasized by the recent report that a number of plants contain less IAA when growing in the absence of epiphytic bacteria, indicating that IAA derived from microbial sources may be important in plant growth processes (Libbert, Kaiser & Kunert, 1969). Yao & Vincent (1969) distinguished between various degrees of curling and reported only the most marked deformations to be specific.

The next occurrence is the softening of the tip of the curled root hair by a pectic enzyme, usually referred to as a polygalacturonase. Here IAA is believed to be involved in the softening process and, by regulation of RNA or protein biosynthesis or both, to control the extension of the root hair.

Subsequently, the bacteria move towards the tetraploid cells of the plant cortex in a non-septate structure, the infection thread. The bacteria are surrounded by a mucilaginous sheath, now known to be of plant origin (Sahlman & Fahraeus, 1963; Dixon, 1964), though controversy exists regarding the derivation of the thread. Dixon (1967) reported that the thread is derived from the plant plasmalemma; Mosse (1964) suggested that it was derived from the endoplasmic reticulum; whilst Dart & Mercer (1963) considered the sheath to be synthesized de novo.

On leaving the thread, the rhizobia become very pleomorphic and stop dividing. This is the bacteroid stage. Meanwhile, the diploid and tetraploid cells of the plant cortex have been rapidly multiplying, giving rise to the ordered radical protuberance, the nodule.

It can be seen from this brief description of nodulation that IAA plays an important role in the early stages. Reduction of the amount of IAA available seriously impedes the progress of infection as has been shown in the case of nitrate inhibition of nodulation. Nitrate is reduced to nitrite by either plant or bacterium and nitrite is known to catalyse the oxidation of IAA (Tonhazy & Pelczar, 1954). Munns (1968b) has shown that delays in nodulation in the presence of nitrate reflect delays in the formation of infection threads. He has further shown that the addition of IAA to lucerne growing on nitrate increases the numbers of nodules on the roots (Munns, 1968c). He considers his data further evidence for the hypothesis "that curling merely coincides with some other acid-sensitive step that is essential for subsequent infection and nodule formation". However, in Trifolium the root hair curling process would seem to be more important than in lucerne since, while in Medicago only 5 p.c. of the infected hairs are associated with nodules (McCoy, 1932), there is in red clover a close correlation between numbers of infected root hairs and nodule counts (Purchase, 1953).

The functions of IAA within the plant are well known. An auxin, it controls plant growth; auxin deficiency results in shortened internodes, restricted leaf expansion, tissue proliferation, loss of apical dominance and the death of apical buds. Wagenknecht

& Burris (1950) showed, using peas and beans, that manganese causes activation of the auxin, while Morgan, Joham & Amin (1966) reported data which supports the hypothesis that manganese catalyses the destruction of IAA oxidase inhibitor(s), thus allowing IAA oxidase to function more actively.

It is therefore possible to suggest a mechanism whereby excess manganese may inhibit, either partially or totally, the process of nodulation, resulting in the decrease in nodule numbers reported here and by Vose & Jones (1963) and Dobereiner (1966).

It is suggested that excess nutrient manganese causes an increase in IAA oxidase activity. This is reflected in a decrease in the levels of IAA, adversely affecting infection thread formation and thus the development of root nodules.

Experimental data to support this hypothesis are not available but it is considered that experiments similar to those of Munns (1968c), in which IAA was added to the plant culture tubes at stages during nodulation, should be undertaken to determine this question.

AGRONOMIC IMPLICATIONS

Natural ecological situations, such as acid hill soils, in which the occurrence of ineffective rhizobia may be limiting nitrogen fixation, are of interest to both agronomists and ecologists. Agronomists are concerned with the establishment and maintenance of Rhizobium populations highly effective on introduced seed. Ecological studies may reveal factors promoting the development of ineffective populations and subsequently show hitherto unknown differences between effective and ineffective strains.

The differences in the response of S. 184 and indigenous hill ecotypes to inoculation by rhizobia (Experimental Work, Part I) are of considerable interest. It has already been suggested that the clover-Rhizobium contribution to the nitrogen status of acid hill soils must be greater than previously appreciated. It must also be concluded that hill rhizobia are more compatible with their natural host than with commercial clover.

Manganese, which in wet acid soils is readily available (Truog, 1946; Lucas & Davis, 1961; Patrick & Turner, 1968) and may be made more available by the acids excreted during the growth of rhizobia (Experimental Work, Part III), has been shown in the laboratory to reduce the symbiotic nitrogen-fixing ability of effective strains. The finding of effective strains in hill soils, however, suggests that manganese does not exert its full effect, either because it is not uniformly available in high concentration or because the native rhizobia have become adapted to its presence. The latter suggestion is illustrated by strain P3R, the symbiotic efficiency of which was not reduced by culture of the strain on

manganese-rich media (Table 16). It is considered that the results of Sherwood (1966) support the idea that high concentrations are necessary for manganese to reduce effectiveness. She, working at pH 5.5 and 400 ppm manganese, found only slight changes in the effectiveness of her strains; at pH 6.5, she reported that manganese had no effect at all.

In view of the low numbers of indigenous rhizobia in acid hill soils (Jones, 1966), inoculation of these areas would at first seem a prerequisite for the successful establishment of white clover. Nevertheless, it is possible for inoculation to fail. Singer, Holding & King (1964) showed that in brown earth soils only a small proportion of effective strains is required to produce a highly effective response. This was confirmed by Sherwood (1967) working with equal numbers of effective and ineffective strains. She found that the isolates from all nodules on S. 100 clover were highly effective; the same results were found when the clover was grown in a gley, pH 5.2. Whilst it is possible that effective strains multiply in the legume rhizosphere more quickly than ineffective, the work of Vincent & Waters (1953) on strain competition indicates that this is unlikely. Their results point to the conclusion that it is the host plant which determines the relative success in nodulation by strains in the rhizosphere irrespective of numbers present. In this respect, it is interesting to note that in the work reported here the effective strains generally nodulated sooner than the ineffective (Appendix 16). The presence in the soil of a few rhizobia effective on commercial seed would, then, appear to indicate that inoculation is unnecessary. If, though, inoculation is decided upon, it is

suggested that Rhizobium strains such as P3R, unaffected by high manganese concentrations, would be more suitable than most strains for use as inocula.

Other features of strain variation must also be considered. Vose & Jones (1963), working with one Rhizobium strain, suggested that manganese affects the Rhizobium-Trifolium symbiosis by reducing the levels of nitrogen fixation. The results presented here show that an important effect of manganese is the reduction of nodulation rate. Even so, variation in the effects of manganese on nodulation of Trifolium by different Rhizobium strains was observed and the nodulation pattern of one ineffective strain was completely unaffected by nutrient manganese concentration (Appendix 16).

The suggestion that manganese reduces the levels of nitrogen fixation cannot be dismissed. It has been shown that, in agar, increased levels of manganese had a variable strain influence on nodulation; similarly it had a variable influence on clover yield (Table 16); but there appears to be no correlation between the two phenomena. The response of white clover to nodulation by Rhizobium trifolii strain 1, for example, was smaller at 28 ppm manganese than at 13 ppm yet nodule numbers were higher at the higher manganese level. This, it would appear, must be due to manganese effects on the nitrogen-fixing systems within the nodule.

The similar survival rates of effective and ineffective organisms do not explain the predominance of less effective rhizobia in acid hill soils. The finding that manganese reduces effectiveness gives credence to the suggestion of Holding & King (1963) that metal toxicity is an important factor in these environments.

With these considerations in mind, it can readily be seen that currently it is difficult, if not impossible, to assess the nitrogen losses to the hill ecosystem caused by manganese toxicity. It must also be admitted that, though Snaydon & Bradshaw (1969) have shown that clovers indigenous to acid soils differ from the non-acid varieties in their responses to nutrient calcium, magnesium and potassium, nothing is known of their ability to withstand high manganese concentrations. The results of this study, however, stress the importance of liming as a factor in the development of good hill pastures. Apart from the effect of increased soil reaction on the herbage value of pastures (Russell, 1961), liming would tend to reduce high manganese levels, thus reducing the delay in nodulation of clover and the tendency of effective strains to become less effective in nitrogen fixation. Liming would also tend to produce a more favourable manganese-calcium balance in the soil, which may be more important than the absolute concentrations of these metals per se (Schmehl, Peech & Bradfield, 1952).

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APPENDIX 1.

Analysis of variance of the response of S. 184 and indigenous hill ecotype clovers to inoculation with Rhizobium trifolii.

Source of variation	d.f.‡	m.s.	
Clover	1	0.000234	NS
Rhizobia	19	0.142839	***
CR interaction	19	0.031807	***
Error	120	0.004897	

‡ Symbols used throughout appendices:

d.f.	degrees of freedom
m.s.	mean square
NS	not significant
*	significant, P = 0.05
**	highly significant, P = 0.01
***	very highly significant, P = 0.001

APPENDIX 2.

Analysis of variance of the comparison of effectiveness of Rhizobium trifolii on S. 184 white clover after growth on media FB and 79.

Source of variation	d.f.	m.s.	
Medium	1	0.049182	*
Rhizobia	8	0.599161	***
MR interaction	8	0.010325	NS
Error	90	0.011358	

APPENDIX 3.

Analysis of variance of the effect of Fe-EDTA on the growth of S. 184 white clover - first cropping.

Source of variation	d.f.	m.s.	
Block	2	0.197708	NS
pH	1	3.967500	***
Liming agent	1	62.107500	***
Fe-treatment	3	19.021389	***
pL interaction	1	20.020833	***
pF interaction	3	1.190278	**
LF interaction	3	2.781389	***
pLF interaction	3	0.516944	NS
Error	30	0.227931	

APPENDIX 4.

Analysis of variance of the effect of Fe-EDTA on the growth of S. 184 white clover - second cropping.

Source of variation	d.f.	m.s.	
Block	2	3.628958	*
pH	1	37.453333	***
Liming agent	1	221.020833	***
Fe-treatment	3	65.618889	***
pL interaction	1	15.187500	***
pF interaction	3	2.010000	NS
LF interaction	3	1.707500	NS
pLF interaction	3	3.983056	*
Error	30	0.976292	

APPENDIX 5.

Analysis of variance of the response of S. 184 white clover to manganese-treated and untreated rhizobia.

Source of variation	d.f.	m.s.	
Manganese	1	0.489781	***
Rhizobia	9	0.182382	***
MR interaction	9	0.055854	***
Error	100	0.008695	

APPENDIX 6.

Analysis of variance of the response of S. 184 white clover to manganese-treated and untreated rhizobia after a further 5 months' growth on manganese-free media.

Source of variation	d.f.	m.s.	
Manganese	1	0.023775	***
Rhizobia	9	0.235515	***
MR interaction	9	0.011133	***
Error	100	0.002068	

APPENDIX 7.

Analysis of variance of the response of S. 184 white clover to manganese-treated and untreated rhizobia (1 subculture).

Source of variation	d.f.	m.s.	
Block	5	0.018379	*
Manganese	1	0.002542	NS
Rhizobia	9	0.345267	***
MR interaction	9	0.002183	NS
Error	95	0.007038	

APPENDIX 8.

Analysis of variance of the response of S. 184 white clover to manganese-treated and untreated rhizobia (1 subculture) after a further 2 weeks' growth on medium FB.

Source of variation	d.f.	m.s.	
Block	5	0.012440	NS
Manganese	1	0.001836	NS
Rhizobia	9	0.285251	***
MR interaction	9	0.001611	NS
Error	95	0.006227	

APPENDIX 9.

Analysis of variance of the effect of manganese on the yield of S. 184 white clover growing in brown earth soil - watering experiment.

Ineffective strains 7AL & 3AS

Source of variation	d.f.	m.s.	
Block	2	1.105833	NS
pH	1	29.925208	***
Rhizobia	1	10.735208	**
Manganese	3	2.882431	NS
pR interaction	1	0.935208	NS
pM interaction	3	0.345764	NS
RM interaction	3	1.361319	NS
pRM interaction	3	0.742431	NS
Error	30	1.141389	

APPENDIX 10.

Analysis of variance of the effect of manganese on the yield of S. 184 white clover growing in brown earth soil - watering experiment.

Effective strains P3 & 1DL

Source of variation	d.f.	m.s.	
Block	2	2.094375	NS
pH	1	93.800208	***
Rhizobia	1	0.000208	NS
Manganese	3	3.869653	NS
pR interaction	1	6.091875	*
pM interaction	3	2.185764	NS
RM interaction	3	0.815764	NS
pRM interaction	3	0.265208	NS
Error	30	1.527264	

APPENDIX 11.

Analysis of variance of the effect of manganese on the yield of S. 184 white clover growing in brown earth soil - watering experiment.

Comparison of effective and ineffective strains

Source of variation	d.f.	m.s.	
Block	2	0.880208	NS
pH	1	17.763333	*
Rhizobia	1	10.830000	NS
Manganese	3	1.812500	NS
pR interaction	1	11.800833	*
pM interaction	3	2.097222	NS
RM interaction	3	2.052778	NS
pRM interaction	3	1.385833	NS
Error	30	2.703319	

APPENDIX 12.

Analysis of variance of the effect of manganese on the yield of S. 184 white clover growing in brown earth soil - incorporation experiment.

Ineffective strains 7 & W19

Source of variation	d.f.	m.s.	
Block	4	3.964969	NS
pH	1	0.731531	NS
Rhizobia	1	0.124031	NS
Manganese	3	4.017865	NS
pR interaction	1	0.569531	NS
pM interaction	3	1.424031	NS
RM interaction	3	1.415865	NS
pRM interaction	3	3.490031	NS
Error	60	2.672335	

APPENDIX 13.

Analysis of variance of the effect of manganese on the yield of S. 184 white clover growing in brown earth soil - incorporation experiment.

Effective strains FA6 & 9CS

Source of variation	d.f.	m.s.	
Block	4	1.253625	NS
pH	1	0.012500	NS
Rhizobia	1	0.040500	NS
Manganese	3	154.934167	***
pR interaction	1	3.280500	*
pM interaction	3	2.593500	*
RM interaction	3	1.634833	NS
pRM interaction	3	0.367500	NS
Error	60	0.724692	

APPENDIX 14.

Analysis of variance of the effect of manganese on the yield of S. 184 white clover growing in brown earth soil - incorporation experiment.

Comparison of effective and ineffective strains

Source of variation	d.f.	m.s.	
Block	4	4.319562	NS
pH	1	0.552781	NS
Rhizobia	1	0.022781	NS
Manganese	3	136.045281	***
pR interaction	1	6.583781	NS
pM interaction	3	6.241115	NS
RM interaction	3	4.152448	NS
pRM interaction	3	3.570781	NS
Error	60	2.965496	

APPENDIX 15.

Analysis of variance of the effect of manganese on the yield of S. 184 white clover growing on nitrogen-free mineral salts agar.

Source of variation	d.f.	m.s.	
Block	5	0.003630	NS
Rhizobia	9	0.067411	***
Manganese	3	0.322458	***
RM interaction	27	0.031272	***
Error	195	0.008183	

APPENDIX 16a.

The effect of manganese on the number of nodules developing on the roots of S. 184 white clover growing on nitrogen-free agar; square root transformation $x' = (x + 0.5)^{0.5}$, where x = no. of nodules/tube of 3 plants.

Rhizobium strain	Mn concentration (ppm)	Days after inoculation 6	9	12	15	18	21	70
1	3	2.32	3.03	3.82	4.02	4.17	4.23	4.33
	13	0.71	0.79	1.83	2.25	2.52	2.66	2.75
	28	1.05	1.13	1.99	2.60	3.00	3.00	3.28
P3	3	1.62	1.87	2.39	2.59	2.74	2.88	3.19
	13	0.85	0.94	2.42	2.73	2.77	2.77	2.92
	28	1.41	1.50	2.33	2.44	2.47	2.51	2.82
1DL	3	1.56	1.85	2.58	2.96	3.19	3.45	3.65
	13	1.24	1.34	2.17	2.34	2.38	2.43	2.57
	28	1.07	1.07	1.18	1.38	1.64	1.64	1.83
P1	3	1.24	1.40	2.08	2.39	2.61	3.08	3.41
	13	0.71	1.23	2.64	3.42	3.67	3.72	3.77
	28	0.79	1.44	1.72	1.84	1.84	2.12	2.43
4AL	3	1.05	1.28	2.77	3.21	3.32	3.49	3.66
	13	0.71	1.03	2.52	2.97	3.19	3.27	3.89
	28	0.71	0.88	1.38	1.58	1.71	1.82	1.97
7AL	3	0.85	1.11	1.98	2.28	2.64	3.01	3.46
	13	0.71	0.71	1.16	1.49	1.89	1.97	2.50
	28	0.71	0.94	1.59	1.89	2.14	2.30	2.30

Data continues in Appendix 16 b.

APPENDIX 16 b.

The effect of manganese on the number of nodules developing on the roots of S. 184 white clover growing on nitrogen-free agar; square root transformation $x' = (x + 0.5)^{0.5}$, where x = no. of nodules/tube of 3 plants.

Rhizobium strain	Mn concentration (ppm)	Days after inoculation: 6	9	12	15	18	21	70
6	3	0.71	0.85	2.50	2.92	3.23	3.29	3.34
	13	0.79	0.93	1.98	2.63	2.83	3.15	3.40
	28	0.99	1.13	1.63	1.72	1.77	1.77	2.06
7	3	0.71	1.10	2.39	2.65	2.99	3.20	3.55
	13	1.17	1.89	2.46	2.92	3.17	3.22	3.57
	28	0.79	0.85	1.40	1.58	2.01	2.09	2.22
2	3	2.03	2.83	4.15	4.39	4.55	4.59	4.61
	13	0.79	0.79	1.41	1.71	1.93	2.10	2.18
	28	0.71	0.79	1.26	1.55	1.71	1.76	1.96
9CL	3	0.71	0.71	1.98	2.30	2.52	2.62	2.91
	13	0.71	0.71	2.23	2.49	2.86	2.92	2.95
	28	0.94	1.26	2.30	2.48	2.63	2.82	2.96

See also Appendix 17.

APPENDIX 17.

Analysis of variance (square root transformation, $x' = (x + 0.5)^{0.5}$) of the effect of manganese on the number of nodules developing on the roots of S. 184 white clover growing on nitrogen-free agar.

Source of variation	d.f.	m.s.	
Block	5	2.120012	NS
Rhizobia	9	5.871818	NS
Manganese	2	90.333851	***
RM interaction	18	11.710895	***
Error A	145	3.738402	
Time	6	109.517895	***
RT interaction	54	0.359265	*
MT interaction	12	2.244895	***
RMT interaction	108	0.368366	**
Error B	900	0.243395	

APPENDIX 18.

Analysis of variance of the effect of manganese on the number of nodules developing on the roots of S. 184 white clover growing in brown earth soil (pH 5.0).

Ineffective strains 7 & W19

Source of variation	d.f.	m.s.	
Block	1	5.761905	**
Rhizobia	1	0.093333	NS
Manganese	2	17.080000	***
RM interaction	2	0.556190	NS
Error A	5	0.139048	
Time	6	2.461905	***
RT interaction	6	0.348889	***
MT interaction	12	0.460000	***
RMT interaction	12	0.038413	NS
Error B	36	0.053968	

APPENDIX 19.

Analysis of variance of the effect of manganese on the number of nodules developing on the roots of S. 184 white clover growing in brown earth soil (pH 5.0).

Effective strains FA6 & 9CS

Source of variation	d.f.	m.s.	
Block	1	2.201905	NS
Rhizobia	1	0.617143	NS
Manganese	2	18.210000	***
RM interaction	2	0.575714	NS
Error A	5	0.374476	
Time	6	6.024127	***
RT interaction	6	0.350476	NS
MT interaction	12	0.405556	NS
RMT interaction	12	0.142381	NS
Error B	36	0.279048	

APPENDIX 20.

Analysis of variance of the effect of manganese on the number of nodules developing on the roots of S. 184 white clover growing in brown earth soil (pH 5.0).

Comparison of effective and ineffective strains

Source of variation	d.f.	m.s.	
Block	1	15.087619	**
Rhizobia	1	0.230476	NS
Manganese	2	2.470000	NS
RM interaction	2	0.920476	NS
Error A	5	0.641905	
Time	6	1.073175	*
RT interaction	6	1.291587	**
MT interaction	12	0.348889	NS
RMT interaction	12	0.231587	NS
Error B	36	0.416190	